

Review

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[Vanyo Mitev](#) *

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Review

Prevention and treatment of COVID-19 and influenza with bromhexine and high doses of colchicine

Vanyo Mitev

Research Institute of Innovative Medical Science and Department of Medical Chemistry and Biochemistry,
Medical Faculty, Medical University-Sofia, Sofia 1431, Bulgaria

* Correspondence: Vanyo Mitev (vmitev@mu-sofia.bg)

Abstract: The Transmembrane Protease Serine S1 subtype 2 (TMPRSS2) and NOD-like receptor protein 3 (NLRP3) inflammasome are the main targets for the prevention and treatment of COVID-19 and influenza. The TMPRSS2 is responsible for the penetration of the SARS-CoV-2 and influenza viruses into the cell, while the hyperactivation of the NLRP3 inflammasome can lead to a cytokine storm, multiorgan damage and death. The correct strategy for preventing illness from COVID-19 and influenza is to preemptively block the TMPRSS2. Preventing the cytokine storm in COVID-19 and influenza is only effective when inhibiting NLRP3 inflammasome. Long-term prophylaxis with the TMPRSS2 inhibitor bromhexine hydrochloride and treatment with high doses of colchicine, able to inhibit the NLRP3 inflammasome, is a very effective, safe and inexpensive method against the spread and complications of COVID-19 and influenza.

Keywords: COVID-19; Influenza; Bromhexine; TMPRSS2; NLRP3 inflammasome; prophylactics; colchicine

1. Introduction

With the discovery of vaccines and antibiotics, major epidemics of plague, smallpox, cholera, tuberculosis, typhoid fever, poliomyelitis are a thing of the past. However, over the past 100 years the world has been ravaged by deadly influenza and corona virus pandemics.

While the number of deaths in the First World War was around 15 million, the H1N1 pandemic in 1918-1920 caused most likely 50 million deaths (according to some even 100 million) with a world population of 1.86 billion [1].

Now, SARS-CoV-2 continues to claim victims, which are already more than 7.09 million deaths according to WHO [2]. It is more likely, however, that the number of victims may be 2-4 times higher [3]. Seasonal influenza caused by influenza viruses A and B (IAV/B) result in 3–5 million severe cases and 290,000–650,000 deaths annually [4]. A Google scholar search shows 4 980 000 articles with the keyword COVID-19 and 992 000 with the keyword Influenza.

The progress in unraveling the pathogenetic mechanisms of influenza and COVID-19 is impressive. In contrast to the great theoretical progress, the treatment recommended by the WHO is very controversial and ineffective.

2. TMPRSS2 - main target for the prevention of COVID-19 and influenza

2.1. TMPRSS2

Of the 178 serine proteases encoded in the human genome (the total number of proteases in man is 699), 138 belong to the S1 family of trypsin-like proteases [5]. Transmembrane Protease Serine S1 subtype 2 (TMPRSS2) is a type II transmembrane protein (with an intracellular NH₂ terminus) which belongs to the human Type II Transmembrane Serine Protease (TTSP) family of trypsin-like membrane-anchored serine proteases. TTSP has 19 members, which are grouped into 4 subfamilies:

HAT/DESC, hepsin/TMPRSS (transmembrane protease/serine S1), matriptase, and corin. The TMPRSS2 is a part of the second subfamily, which has seven members - hepsin, TMPRSS2, TMPRSS3, TMPRSS4, TMPRSS5/spinesin, MSPL (mosaic serine protease large-form), and enteropeptidase [6]. As a serine protease, TMPRSS2 is involved in the cleaving peptide bonds of proteins that have serine as the nucleophilic amino acid within the active site [7].

TMPRSS2 is expressed in many epithelial tissues, including prostate (expressed several times higher than in any other tissue), lung (trachea, bronchus), breast, kidney, gastrointestinal tract (small intestine, colon), pancreas, bile duct, thymus, ovary and salivary glands [8-10].

Very little is known about the physiological role of the TMPRSS2. TMPRSS2 regulates the Na⁺ current by cleaving sodium channels, thereby facilitating ion passage [11-12], but it is not required for normal development, and does not cause pathological changes as demonstrated in TMPRSS2 knockout mice [13].

On the other hand, there is considerable evidence of the involvement of TMPRSS2 in various pathological processes.

v-ets erythroblastosis virus E26 oncogene-homolog (*ERG*) is an oncogene encoding a family of transcription factors - key regulator of cell proliferation, angiogenesis, differentiation, inflammation and apoptosis [14]. The *TMPRSS2:ERG* fusion gene is common in prostate cancer, occurring in about 50% of cases and driving early transformation and metastasis [15]. TMPRSS2 has been implicated in other diseases such as hepatitis C [16], chronic hepatitis B and hepatocellular carcinoma [17], ulcerative colitis [18], oncological diseases, including colorectal, gastric, prostate, pulmonary, and hepatic cancers [19].

2.2. Role of TMPRSS2 in Influenza and COVID-19 Infections

2.2.1. Influenza

Influenza viruses (IV) are classified into four categories: IAV, IBV, ICV and IDV. IAV and IBV are the predominant types, causing human seasonal epidemics. IAVs are stratified into subtypes, according to the specific configurations of their surface hemagglutinin. There is a diversity of 18 hemagglutinin (HA) and 11 neuraminidase (NA) different protein subtypes, which allows 144 potential variants [20]. In contrast, IBVs are categorized into distinct lineages: B/Yamagata and B/Victoria [21].

HA and NA are the major IV glycoprotein antigens (Fig. 1). HA is responsible for both attaching the virus to cell surface receptors and the membrane fusion, while NA mediates the release and dissemination of virions from infected cells [22]. HA0 is the inactive precursor of HA. The crucial step for virus infectivity is the cleavage of HA0 by TMPRSS2, resulting in two functional subunits with fusion competence: HA1 and HA2 [23]. TMPRSS2 is the major HA-activating protease of IAV in primary human bronchial cells and of both IAV and IBV in primary human type II pneumocytes [24,25]. HA is cleaved by TMPRSS2 in the Golgi apparatus during assembly [26]. Currently, prevalent IAV subtypes in human circulation include H1N1 and H3N2 [27].

Inhibition of TMPRSS2 would also be useful in battling respiratory parainfluenza viruses as it is an activating protease for them as well [28].

It should be noted that the dependence on TMPRSS2 is different for the different subspecies of IAV. It is bigger for H1N1/1918 and H13, while recombinant IAVs carrying H12 or H17 were not affected by TMPRSS2 knockdown [29].

2.2.2. SARS-CoV-2

SARS-CoV-2 enters into cells via two distinct pathways: one is mediated by TMPRSS2 at the cell surface (Entry via plasma membrane/ Early endocytosis) and the other done by cathepsin L/B in the endosome (Entry via endosomal pathway/Late endocytosis). TMPRSS2 could act in both early and late endosome entry processes [30,31]. TMPRSS2 also plays a critical role in the proteolytic activation of SARS-CoV, MERS-CoV and SARS-CoV-2 [29].

Although TMPRSS2 and cathepsin L/B are the main proteases responsible for cell penetration, the SARS-CoV S protein can be cleaved as well as by HAT, MSPL, DESC1 and Factor Xa [32-34]. However, TMPRSS2 serves as a major cofactor rather than cathepsins for SARS-CoV-2 cell entry [35-37].

TMPRSS2 has been implicated in the regulation of the SARS-CoV assembly in the Golgi apparatus and the release of the mature virus from the host plasma membrane [26,38-40]. TMPRSS2 is also responsible for the viral spread in the infected host [41].

Thus, spike cleavage by TMPRSS2 takes place in the plasma membrane (at cell entry), in Golgi membrane during assembly, and during virus release and spread. All these facts make TMPRSS2 an attractive target for treating both COVID-19, as well as influenza and other infectious diseases.

2.3. Inhibitors of TMPRSS2

2.3.1. Camostat Mesylate

Great hopes were placed on the TMPRSS2 inhibitor Camostat Mesylate/CM (used to treat pancreatitis and reflux esophagitis) for the treatment of COVID-19 [42]. Over 10 prestigious clinical trials have been launched to investigate the effectiveness of CM in COVID-19 and graduated with disappointing results [43-46]. This failure is not difficult to predict, because CM is administered only after the virus has already entered the cell [31].

2.3.2. Bromhexine hydrochloride

Another TMPRSS2 inhibitor is bromhexine hydrochloride (BRH), an over-the-counter, non-invasive, effective, with proven safety, available globally, inexpensive and well-tolerated medicine. Since 1963, the mucolytic cough suppressant BRH has had a long history of use in respiratory tract disorders. In addition, it has been shown to have anti-inflammatory effects, thus reducing swelling and irritation in the respiratory tract [31,47,48].

The conflicting data about the role of BRH in the prevention and treatment of COVID-19 [47-56] can be explained in just a few words - timing and mode of application. With this in mind, we hypothesized that TMPRSS2 inhibition by BRH would be most effective when taken prophylactically or administered immediately after a contact with a sick or infected person (post-exposure prophylaxis).

We recently published our data analyzing the results of 125 people who took BRH prophylactically during the COVID-19 pandemic [31]. The effect of BRH is best when given continuously for prophylaxis during peaks of contagion in the wave of COVID-19. Then the probability of infection drops sharply, and if a disease does occur, it proceeds mildly. BRH is also effective when given by inhalation for post-exposure prophylaxis. When COVID-19 manifests itself clinically, the efficacy of BRH drops sharply because the virus is already in the cell. However, BRH inhalations are useful because they limit the spread of the virus and have an antiinflammatory and possibly antiviral effect [31,57].

3. NLRP3 inflammasome - main target for treatment of COVID-19 and influenza

3.1. Structure and distribution of NLRP3 inflammasome

The NLRP3 inflammasome is a multi-protein complex, the "cornerstone" of the innate immune system, comprising of the NLRP3 sensor protein, an adaptor protein ASC [apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)], and an effector protein (pro-caspase-1). The NLRP3 protein, aptly named "architectural marvel", consists of three distinct domains: a C-terminal leucine-rich repeat (LRR) sensor domain, a central NACHT domain responsible for oligomerization and an N-terminal pyrin (PYD) domain responsible for protein-protein interactions [58,59]. This intricate structure allows NLRP3 to function as a molecular switch, transitioning from an inactive to an active state in response to cellular danger signals [60].

NLRP3 inflammasome activation is most widely studied in myeloid cell types (macrophages, monocytes, neutrophils and dendritic cells), but it is also expressed in nearly all cell types, and importantly, lung fibroblasts and bronchial epithelial cells [61,62].

3.2. Activation of NLRP3 inflammasome

In the absence of activating signals, the inactive oligomeric NLRP3 is in a complex with HSP90 and SGT1 in the cytoplasm and pro-IL-1 β is not constitutively expressed in resting macrophages [63].

NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) upregulates the expression of NLRP3, which under resting conditions exists at concentrations that are inadequate for initiating inflammasome activation (supramolecular complex) and pro-IL-1 β maturation [64,65].

The canonical activation of NLRP3 inflammasomes requires two signals: signal 1 (priming) is provided by TLR (Toll-like receptors), IL-1 and TNF (tumour necrosis factor) receptors activation, which will induce the activation of NF- κ B and the up-regulation of inactive pro-IL-1 β , pro-IL-18 and inflammasome components [66,67].

According to others, priming signals do not affect the expression levels of pro-IL-18, ASC and pro-caspase-1 [59,68]. Inflammasome activation and inflammatory 'strength' is modulated via transcriptional regulation of signal 1 [66].

Signal 2 is provided by various stimuli, including K⁺ efflux, extracellular ATP activation of the P2X7 receptor, pore-forming toxins, crystalline substances, cellular stress signals as reactive oxygen species (ROS), influenza virus M2/PB1-F2 proteins, ZBP1 (Z-DNA binding protein 1) and RNA viruses. These events lead to the oligomerization of the inflammasome complex through ASC interaction with inactive pro-caspase 1, which is activated. The activated caspase 1 cleaves pro-IL-1 β /IL-18 to IL-1 β /IL-18 and gasdermin D, which induces pore formation in the plasma membrane and consequently IL-1 β /IL-18 release and pyroptotic cell death [66]. IL-1 β induces the production of IL-6, another abundantly detected cytokine in severe COVID-19 patients [69,70]. IL-6 stimulates the release of various acute phase proteins, such as the hepatic factors C-reactive protein and ferritin, which are associated with poor prognosis [71,72]. IL-18 has been linked to ferritin production [73] and is another line of evidence for NLRP3 in severe COVID, as its level is significantly higher in symptomatic patients and is increased in accordance with disease severity. IL-1 β itself has been associated with severe COVID-19 [74,75]. In macrophages, the priming signal (signal 1) is mandatory for inducing NLRP3 inflammasome activation [68].

SARS-CoV-2 can activate NLRP3 inflammasome by ORF3a (open reading frame 3a) [76,77]; Nucleocapsid [78]; NS6 (non-structural protein 6) [79]; Spike [80,81]; NS5 [82]; viroporin E [77]; viroporin M [83].

Many other viruses can activate the NLRP3 inflammasome such as SARS-CoV, Influenza, Parainfluenza, Adenovirus, Bocavirus, Metapneumovirus, Respiratory syncytial virus, Rhinovirus [84].

The role of activated NLRP3 is to limit the infection by SARS-CoV-2 [85]. When the NLRP3 inflammasome is properly activated, IL-1 β production and the activation of the immune system help to resolve the infection. However, inflammasome inhibition by the virus, leads to easy replication and spread, resulting in worse disease prognosis. The same applies to its hyperactivation, leading to cytokine storm, multiorgan damage, and often death [85]. Thus, the regulation of the NLRP3 complex and pro-IL-1 β expression are critical to limit inflammasome function [66].

3.3. Regulation of NLRP3 inflammasome

NLRP3 inflammasome is subjected to strict post-translational regulation including phosphorylation/dephosphorylation, ubiquitination/deubiquitination, SUMOylation, S-nitrosylation, ADP-ribosylation, acetylation, O-GlcNAcylation, nitration of Tyr861, glycosylation, palmitoylation [for. Refs see 67].

For example, negative NLRP3 inflammasome regulators are several ubiquitin ligases, including TRIM31 [86], MARCH7 [87], RNF125 [87], CBL-b [88], FBXL2 [88], ARIH2 [89] and Cullin1 [90].

While most ubiquitin ligases are negative regulators of NLRP3 inflammasome, some as Pellino2 [91] and TRAF6 [92] promote its activation.

In most cases the action of the deubiquitinating enzymes such as BRCC3 [93], USP7 [94], USP47 [94], leads to positive regulation of the NLRP3 inflammasome. However, A20 functions as NLRP3 inflammasome negative regulator [95].

Phosphorylation/dephosphorylation plays a critical role in the NLRP3 inflammasome regulation, with different phosphorylation sites exerting sometimes opposing effects [96]. Positive regulators are protein kinase D [97], protein phosphatase 2A (PP2A) [88], c-Jun N-terminal kinase 1 (JNK1) [98], protein tyrosine phosphatase nonreceptor 22 (PTPN22) [99], whereas negative regulator is PKA [89] while AKT phosphorylation has a dual role [100].

The activated NIMA-related kinase 7 (NEK7) can induce NLRP3 conformational change, leading to disruption of its inactive double-ring structure [90].

Another negative regulator is type I interferon (IFN) expression believed to prevent the induction of a hyperinflammatory NLRP3 inflammasome activation [101,102]. IFN-I is required for the recruitment of pro-inflammatory monocytes and macrophages to the infected lungs [103]. However, the early use of IFN α decreased mortality, whereas late use of IFN α increased mortality [104].

People with severe COVID-19 have symptoms of systemic hyperinflammation, mediated by a rapid release of inflammatory molecules, especially inflammatory cytokines such as interleukin (IL)-1 β , IL-18, IL-6 and tumor necrosis factor- α , and the protein Gasdermin D (GSDMD) which is a marker of inflammatory cell death [105].

4. Clinical course of influenza and COVID-19

4.1. FLU

Clinically, the flu progresses as follows: After an incubation period of an average of 2 days, the flu begins acutely and day 1 to day 3 is characterized by worsening of the symptoms. FLU symptoms usually peak between days 2 and 4. A slight decline in induction is noted on day 4, most people feel better after 5–7 days and recover on day 8. In this case, there is a normal response to the NLRP3 inflammasome. However, in some people the disease rebounds on day 7 to levels similar to the onset of infection, suggesting two ‘waves’ of inflammation, leading to a ‘feed-forward’ inflammatory loop [106]. This development of the disease corresponds to hyperactivation of the NLRP3 inflammasome with excessive IL-1 β and IL-18 maturation, with subsequent increase in levels of IL-6 and other cytokines (CS).

Thus, the NLRP3 inhibition during the first five days after the onset of IAV infection can be detrimental, while it proves beneficial in the later stages (the period from the seventh day to the ninth day) [106].

4.2. COVID-19

Clinically, COVID-19 progresses as follows: After an incubation period of an average of 2-14 days, day 1 is characterized by mild symptoms, which escalate during day 2 to day 7. Days 8-10 are critical and are followed by either improving or worsening symptoms. Recovery occurs between days 15 and 21. If the NLRP3 inflammasome response is normal, recovery should occur after the critical 8-10 days. However, after hyperactivation of NLRP3 inflammasome, the cytokine storm leads to multiorgan damage, microthrombosis, and death. LDH level on day 8 is the strongest predictor of in-hospital mortality in COVID-19 inpatients and the second one is the decreased number of peripheral blood lymphocytes [107]. Thus, the inhibition of NLRP3 inflammasome is mandatory to prevent cytokine storm.

In short, the incubation period of COVID-19 is longer, and it is more contagious than the flu, the onset of symptoms is milder, the recovery is slower, complications due to NLRP3 inflammasome hyperactivation are more common, and mortality is higher. What is the common clinical feature between COVID-19 and the flu are the critical days during the development of the symptoms (Tabl. 1).

5. Strategies for dealing with COVID-19 and the flu - which one is the winner?

5.1. Antivirals and inhibitors of individual cytokines

The best strategy would be to prevent the virus from entering the host cells.

For a number of reasons, vaccines are not effective enough against either COVID-19 or the flu. COVID-19 vaccines do not appear to be effective against secondary infection. What's more, significantly high number of vaccinated individuals had a secondary encounter and subsequent infection with COVID-19 [108], which is explained by their "high-risk" activities, such as avoiding social distancing, engaging in more-frequent public activities [109].

Drug-based blocking of the virus entry into the cell may be used both as primary and post-exposure prophylaxis. As we emphasized above, the effect of TMPRSS2 inhibitors drops dramatically after the onset of the disease, as the viruses are already in the cells and multiplying and spreading intensively. This also explains the collapse of clinical trials with the TMPRSS2 inhibitor camostat mesylate [43-46].

The WHO and Big Pharma strategy of using antivirals and inhibiting individual cytokines is not productive, because there is no direct correlation between viral load and NLRP3 inflammasome hyperreaction [110]. Furthermore, inhibiting individual cytokines in the presence of ongoing NLRP3 inflammasome hyperactivity is pointless. [48,57,110].

It is clear that the main cause of complications in COVID-19 and influenza is the hyperactivated NLRP3 inflammasome. Its regulation is multifactorial. How all these positive and negative regulatory factors act spatially and temporally, what their cumulative effect is, and why around days 8 and 10 the NLRP3 inflammasome becomes hyperactivated in complicated cases remains a mystery. Moreover, the regulation of the normal NLRP3 inflammasome response, day by day, during the illness is unclear. It is mandatory to inhibit NLRP3 inflammasome when clinical signs of impending complications are present.

5.2. Inhibition of TMPRSS2 and NLRP3 inflammasome

Our strategy for the prevention and treatment of Covid-19 and influenza is based on the following facts:

1. SARS-CoV-2 and influenza A and B viruses enter the cell mainly through TMPRSS2.
2. Bromhexine inhibits TMPRSS2.
3. The effect of BRH is best when taken prophylactically, not after the viruses have already entered the cell.
4. Post-exposure prophylaxis with BRH is also very effective, especially when done by inhalation.
5. Complications in both influenza and COVID-19 are due to a NLRP3 inflammasome hyperreaction, which causes a cytokine storm.
6. Colchicine accumulates in myeloid cells, which explains its NLRP3 inflammasome inhibitory effect at high doses.
7. Inhibition of the NLRP3 inflammasome prevents cytokine storm and normalizes cytokine levels.
8. Higher doses of colchicine have been used in the past and are completely safe, provided the rules for administering colchicine are being followed.

With a series of clinical cases, we demonstrated the life-saving effect of high doses of colchicine in critically ill patient [111], those with high levels of obesity [112-114], and the unique case of

recovery of a 101-year-old patient infected with COVID-19 in intensive care after major surgery [115]. It is very demonstrative that four patients who mistakenly took more than 12.5 mg of colchicine recovered quickly and completely after discontinuing all therapy [47,116].

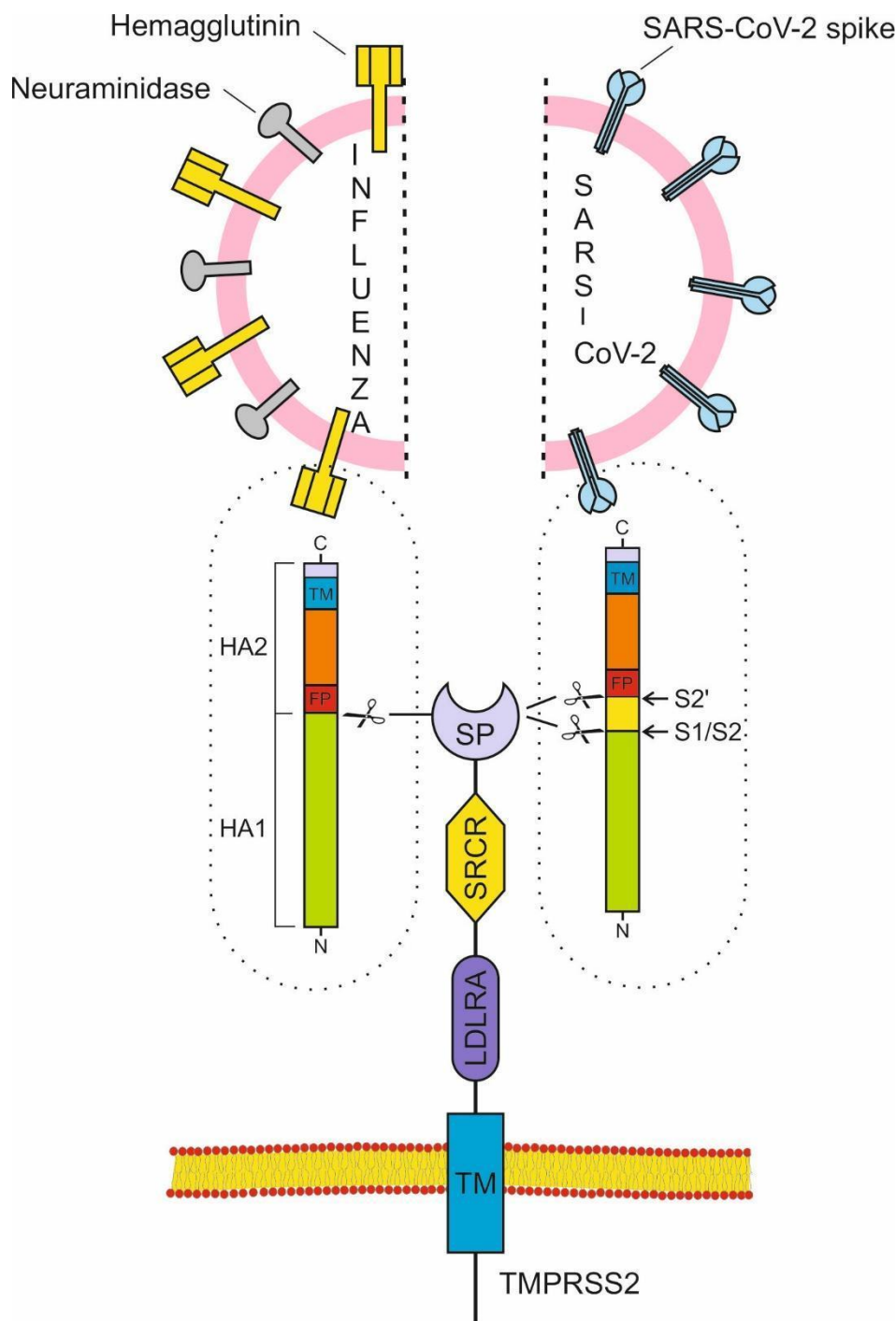
Studies on 795 inpatients treated with high doses of colchicine reduced mortality by 2 to 7 times, in direct proportion to increasing doses [117,118]. Outpatients' high-dose colchicine treatment practically prevents hospitalizations and demonstrates reverse relationship with hospitalization [108]. The maximum loading doses we use of up to 5mg of colchicine (0.045mg/kg) are completely safe [48,57,119].

The effect of BRH is best when given continuously for prophylaxis during peaks in contagion in the wave of COVID-19. As a result the probability of infection drops sharply, and if a disease does occur, it proceeds mildly [47,120].

The epidemic situation in Bulgaria for the winter of 2025 is dominated by influenza viruses A(H3N2 and H1N1pdm09) and influenza viruses B/Victoria. We are monitoring several hundred people taking BRH prophylactically. The results will be summarized at the end of the epidemic, but so far none of those taking prophylactic BRH have fallen ill. Our preliminary results were reported at a national conference [121] and published [122].

Conclusion

Prophylaxis with bromhexine and treatment with high doses of colchicine is a very effective and inexpensive method against the spread and complications of COVID-19 and influenza.



Hemagglutinin (HA); Fusion Peptide (FP); Transmembrane domain (TM); Low-Density Lipoprotein Receptor class A (LDLR-A); Scavenger Receptor Cysteine-Rich (SRCR); Serine Protease domain (SP).

Figure 1. Dependence of Influenza and SARS-CoV-2 viruses on TMPRSS2.

Table 1. Viral, Clinical and Laboratory Comparison Between Influenza and COVID-19.

	Influenza	COVID-19
Viruses	Spanish influenza in 1918 (H1N1), Asian influenza in 1957 (H2N2), Hong Kong influenza in 1968 (H3N2), Russian influenza in 1977 (H1N1)	SARS-CoV-2, 2019
Virus family	Orthomyxoviridae	Coronaviridae

Viral nucleic acid	Negative sense single-stranded RNA	Positive sense single-stranded RNA
Vulnerable contingent	children and adults H1N1 influenza in 1918 - young people	adults
Incubation period	2 days	2-14 days
R0	1.4-2.8	5.7
Tropism	Respiratory tract epithelium	Multiple organs
Host receptor	α 2,6 sialic acids	ACE2
Viral proteins required for fusion	HA	S
Critical protease	TMPRSS2	TMPRSS2
First symptom	Cough	Fever
Contagiousness	One day before the onset of symptoms and up to five to seven days after	48 hours before the onset of symptoms and up to 10 days after
Most contagious	The first three days after the onset of symptoms	1-2 days before the onset of symptoms
Viral load peak	2 day	Early at the onset of symptoms, now in a highly immune adult population - 4 day
Worst Days Alert	7-9	8-10
Cause of cytokine storm	NLRP3 hyperactivation	NLRP3 hyperactivation
IL-6/ IL-1/ IL-18/ D-Dimer/ Hypercoagulative state/ DIC/ Endotheliopathy / Ferritin/ Activated macrophages/ High neutrophil to Lymphocyte ratio/ Immunothrombosis, LDH	++/++/+/++/++/++/ +++/++/+/++/ +++/+++	+++/+++/+++/++/+++/ ++/+++/++/+++/++/+++
Radiological findings	Multilobe consolidations	Ground-glass opacities
Need for hospitalization	5.6%	20%
Need for intubation	4.8%	10%–15%
Mortality	0.13%–1.36%	1.40%–3.67%
Prophylaxis	BRH	BRH
Post-exposure prophylaxis	Inhaled BRH	Inhaled BRH
Cytokine storm treatment	High colchicine doses	High colchicine doses

ACE2, angiotensin-converting enzyme metallopeptidase 2; COVID-19, coronavirus disease 2019; HA, hemagglutinin; S-spike protein, cytokine storm (CS).

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