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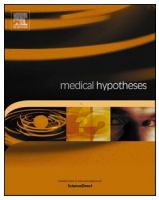

Use of natural cysteine protease inhibitors in limiting SARS-Co-2 fusion into human respiratory cells

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ABSTRACT

Specific antibodies that humans acquire as a result of disease or after vaccination are needed to effectively suppress infection with a specific variant of SARS CoV-2 virus. The S protein of the D614G variant of coronavirus is used as an antigen in known vaccines to date. It is known that COVID-19 disease resulting from infection with this coronavirus can often be very dangerous to the health and lives of patients. In contrast, vaccines produce antibodies against an older version of the protein S-D614G (January 2020) and therefore have difficulty recognizing new variants of the virus. In our project we propose to obtain specific and precise antibodies by means of so-called controlled infection against specific infectious variants of the SARS-CoV-2 virus "here and now". Currently, several variants of this pathogen have already emerged that threaten the health and lives of patients. We propose to reduce this threat by partially, but not completely, blocking the fusion mechanism of the SARS-CoV-2 virus into human respiratory cells. According to our plan, this can be achieved by inhibiting cathepsin L activity in respiratory cells, after introducing natural and non-toxic cysteine protease inhibitors into this area. We obtain these inhibitors by our own method from natural, "human body friendly" natural resources. We hypothesize that blocking cathepsin L will reduce the number of infecting viruses in cells to such an extent that COVID-19 developing in infected individuals will not threaten their health and life. At the same time, the number of viruses will be sufficient for the body's own immune system to produce precise antibodies against a specific version of this pathogen.

Introduction

The SARS-CoV-2 coronavirus emerged in December 2019 in China and has already led to the deaths of more than six million people, infecting several hundred million on all continents. The COVID-19 pandemic has further disrupted the lives of billions of people around the world. The virus enters the human body through the nose, mouth or eyes and attaches to airway cells whose membranes have the protein receptor ACE2. The latter is a metalloproteinase that converts angiotensin 2, which regulates fluid pressure in the human body. After fusion, the coronavirus, already inside the cell, releases its genetic material in the form of RNA. The infected cell reads the information encoded in this genetic material and begins to produce proteins that are used to make copies of descendant viruses. Each infected cell releases a significant

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number of virus particles before it eventually breaks down on its own. New viruses infect more cells and, entering the transmission phase, leave the body when coughing, sneezing, or inhaling saliva aerosols. New viruses must always activate their surface protein S before infecting new cells [1–4]. Small particles of infectious secretions can not only come into contact with human mucous membranes but also adhere to surfaces such as clothing, cell phones, doorknobs, window panes, etc. The virus can remain infectious on these surfaces for several hours to several days (depending on surface structure and environmental conditions), from where it can be transmitted to hands and from hands to mouth, indirectly infecting people. For this reason, it is recommended - especially for patients diagnosed with COVID-19 - to wear masks to prevent the release of viruses and thus reduce environmental contamination. It is also important to remove SARS-CoV-2 viruses and destroy their lipid envelope by washing hands and other surfaces with detergents or solvents, which leads to destruction of the viral envelope, thereby inactivating the virus before it re-enters cells. The use of mouth masks and disinfection is expected to prevent the rapid spread of pandemic COVID-19, leading to a flattening of the incidence curve for the intensity of infection cases [5–7]. The COVID-19 pandemic has become one of the greatest challenges to modern medicine. Although three years have passed since its outbreak, health care systems have very few drugs to contain it. Attempts to repurpose known drugs to treat other pandemics, including influenza, have also failed. Various potential treatments have been attempted, but most are in the preliminary stages of research. An undoubted success is the approval and introduction into treatment of new vaccines against SARS-CoV-2 that enhance the body's ability to neutralize the infecting pathogen [8,9]. Since its inception, the COVID-19 pandemic has motivated the scientific community to prepare vaccines to safeguard the health of billions of people worldwide as quickly as possible. Currently, most vaccines are designed to elicit antibodies that neutralize the virus' S protein, thereby inhibiting virus identification and viral particle uptake by binding to the human ACE2 receptor, which blocks cell infection. S proteins have two primary tasks: 1) they mediate the binding of the virus to the ACE2 receptor on the host cell surface and 2) they facilitate the entry of the virus into human cells. Since the beginning of the pandemic, many papers have been published evaluating the efficacy of antibodies produced by vaccination or after recovery from COVID-19 in neutralizing the ACE2 ↔ S-SARS-CoV-2 complex [10,11]. The SARS-CoV-2 virus, which first emerged in China as variant D614, is constantly mutating to create new variants. Emerging mutations can lead to inefficiencies in both diagnosis and treatment of infected patients. The production of the first vaccines created the opportunity to quickly control the COVID-19 pandemic. However, two major problems have arisen concerning: first, the persistence of antibodies produced both after vaccination and in patients who have recovered, and second, the efficacy against the new, changing variants of the SARS-CoV-2 virus created by mutations, in most cases, of the S protein. It has been confirmed that antibodies in patients who have recovered from SARS-CoV-2 infection or in vaccinated individuals can protect the human body for 6 to 10 months. It has also not been definitively clarified whether new variants of the SARS-CoV-2 virus can be effectively neutralized by antibodies produced against its original D614G variant, which is the model for currently available vaccines. According to estimates, even if the effectiveness of antibodies in neutralizing the mutant coronavirus drops to 60 %, they may protect the body from secondary infection or mitigate the course of the disease [12,13]. However, the problems presented are not limited to the variants that have emerged so far, but also to those that may emerge in the future. It cannot be excluded that some of the new variants will have higher mortality and infectivity. There are also reports of some insufficient efficacy of vaccines based on the Wuhan virus strain [14–16]. This may be due to the quality of antibodies acquired after vaccination or after recovery from the disease. In both cases, antigenic stability ranges from 6 to 12 months [17,18]. As confirmed, some new variants of the SARS-CoV-2 virus may be much more dangerous to human health and life than the prototype D614G virus. The emergence of new variants of this virus was observed repeatedly during the COVID pandemic. Variants of concern, such as delta and omicron, are versions of the SARS-CoV-2 virus that have mutated. These mutations may give the new SARS-CoV-2 virus a genetic advantage-for example, the delta variant is associated with a higher risk of disease than the original Wuhan strain, while omicron is more infectious [19,20,21,22]. COVID 19 vaccination can be compared to influenza vaccination, which requires annual booster doses. These vaccines are developed each year based on the currently infectious virus strains that infect the environment. However, the research leading to the production of a vaccine takes several months, and during this time there is a risk that new variants of influenza viruses will emerge [23]. Studies in Israel [24–26] have confirmed that patients who recover from infection with the delta variant of SARS-CoV-2 virus maintain relatively elevated levels of specific antibodies to the infecting coronavirus for up to 12 months. Individuals vaccinated with BioNTech-Pfizer's RNA vaccine cease to exhibit maximal quantitative and qualitative antibody levels as early as several months after the second dose of the vaccine. It also found that individuals vaccinated in early 2021 were nearly twice as likely to develop a severe form of SARS CoV-2 virus disease as similar individuals vaccinated less than a month earlier. In addition, new variants of SARS-CoV-2 virus may be insensitive to the immune response induced by a vaccine containing coronavirus S protein D614G as antigen. In contrast, the Moderna vaccine (mRNA-1273) demonstrated efficacy of ~ 94 %. Induced antibody activity against various variants of SARS-CoV-2 virus persisted 6 months after the second dose, although at a reduced level compared to peak activity. More than half of the patients also retained neutralizing activity against the B.1.351 variant at the last time point tested. The neutralizing capacity of sera from humans who received Moderna mRNA-1273 vaccine was evaluated. The emergence of SARS-CoV-2 variants with mutations in the spear protein in isolates from the United Kingdom (B.1.1.7) and South Africa (B.1.351), led to reduced neutralization from convalescent sera using pseudovirus neutralization (PsVN) assays and resistance to certain monoclonal antibodies. There was no significant effect on neutralization against variant B.1.1.7. However, reduced neutralization was shown against mutations present in B.1 0.351, but significant neutralization against the full variant was still confirmed after immunization with the mRNA-1273 vaccine. Induced antibody activity against SARS-CoV-2 variants persisted 6 months after the second dose, albeit at a reduced level compared with peak activity, with more than half of the patients maintaining neutralizing activity against B.1.351 at the last time point tested [27,28]. The ACE2 protein, which conditions SARS-CoV-2 virus binding, regulates blood pressure as well as salt and fluid balance in the human body. Therefore, it is present in many organs, including cells of the nose, throat, lung, heart, kidney, liver, brain, and adipose tissue, and cannot be considered a target for future COVID-19 therapies. For some coronaviruses, including HCoV-NL63, SARS-CoV-1, and SARS-CoV-2, it serves as a site of entry into the cell. At the cell surface, it facilitates endocytosis and translocation of the virus into endosomes [29,30]. It has been suggested that the enzymes responsible for the activation phase of the fusion determining peptides in the S protein of the SARS-CoV-2 virus during cell entry are two proteolytic enzymes (in the cell): the transmembrane serine protease 2 TMPRSS2 and the cysteine cathepsin L [31]. Inhibition of both of these enzymes was observed to block the ability of SARS-CoV-2 to fuse and synthesize in cells. We also compared the mechanisms of cell infection by other coronaviruses, including SARS-CoV-1, MERS or influenza viruses, confirming that cysteine cathepsins L and/or B play an important role in their infection. It has been observed that when the ACE2 receptor protein "catches" SARS-CoV-2 coronaviruses, TMPRSS2 and cysteine cathepsin L together can expose specific peptides in their S protein that are responsible for the downstream phase associated with viral fusion into the cell [31–33]. It is also known that cathepsins B / L activate viral fusion in human cells not only of influenza viruses or cold viruses, but also of Ebola, Hendra, Nipah and probably other viruses. As suggested, blocking such cysteine peptidases,

including B/L cathepsins, bodes well for attempts to control the dangerous diseases caused by these viruses [34,35]. The ability to control the activity of human serine and cysteine proteases, which directly activate the S protein of SARS-CoV-2 virus, offers hope for the successful development of new therapies for the treatment of COVID-19. During in vitro and in vivo studies in transgenic animals, we found that the level of cysteine cathepsin L was higher after SARS-CoV-2 infection and was positively correlated with the course and severity of the disease, and overexpression of this enzyme was correlated with the amount of virus in infected cells. Therefore, this enzyme has been described as a promising target for developing new drugs against COVID-19 [36–38]. The most advanced studies use the control of SARS-CoV-2 virus fusion by blocking the serine protease TMPRSS2, which has been achieved by known specific serine inhibitors, i.e. camostat and its derivatives. This drug (camostat) is approved for the treatment of pancreatitis in Japan. Aprotinin, a known peptide serine protease inhibitor, has also had some hope. It was initially thought to be a potential anti-influenza drug, but it has not been approved for treatment outside Russia. The reason is its strong effect on blood clot formation, which caused it not to be approved first for influenza treatment and now for COVID-19 treatment [39].

The hypothesis

We propose to reduce the risk of SARS-CoV2 virus infection by partially, but not completely, blocking the fusion mechanism of this virus into human respiratory cells. According to our plan, this can be achieved by inhibiting the activity of cathepsin L in respiratory cells, following the introduction of natural and non-toxic cysteine protease inhibitors into this area. We obtain these inhibitors by our own method from natural, "human body-friendly" natural resources. We hypothesize that blocking cathepsin L will reduce the number of infecting viruses in cells to such an extent that COVID-19 developing in infected people will not threaten their health and life. At the same time, the number of viruses will be sufficient for the body's own immune system to produce precise antibodies against a specific version of this pathogen.

Evaluation of the hypothesis

In 2012, we developed a method to isolate inhibitors from natural raw materials. However, cystatin from egg white showed too low stability to be used in technologies without specific stabilizers. [40]. In contrast, in 2020 [41], we developed a method to isolate cysteine peptidase inhibitors from both chicken egg white and selected plants or other natural raw materials. Using this method, we isolated both inhibitors with molecular weight above 10 kDa and those with low molecular weight (<3 kDa). Only cystatin from egg white was found to be unstable, whereas inhibitors of cysteine peptidases from plants with a molecular weight above 10 kDa, including inhibitors obtained from Japanese knotweed (Fallopia japonica), were stable up to 24 months after isolation. Low molecular weight (<3 kDa) inhibitors, including inhibitors obtained from both egg white and "human-friendly" plants, showed similarly high stability. They inhibited both the activity of exogenous cysteine peptidases secreted by pathogenic microorganisms, such as gingipain (from P. gingivalis), and the activity of cysteine cathepsin B. Furthermore, we observed that the inhibitors we isolated can be transferred in aqueous aerosols produced in nebulizers without losing their primary activity against cysteine proteases. Therefore, we propose to use this method to deliver the isolated inhibitors to the oral cavity and airways. We have previously shown that these inhibitors have very low toxicity. When the COVID-19 pandemic emerged, we decided to propose to exploit the properties of our isolated natural inhibitors by inhalation, oral sprays of aqueous aerosols containing such inhibitors and sprays or oral soluble tablets, etc. [42]. In our study, the inhibitors isolated from Japanese knotweed were not toxic at concentrations for their potential use and can be digested in the human body. We obtained preliminary results when studying influenza viruses. We verified that inhibitors with a molecular weight greater than 10 kDa blocked the fusion of AH1N1 viruses with cells, and low-molecular-weight cysteine peptidase inhibitors (<3 kDa) blocked the hemagglutinin of influenza viruses, which we confirmed using the hemagglutination inhibition (HI) assay. The low molecular weight inhibitors showed activity similar to antibodies obtained by influenza patients or influenza vaccinated patients. For this reason, we refer to these inhibitors as "antibody-like" [41,42]. Based on findings from other teams working on influenza virus infections, we suggest that interacting with these enzymes in the respiratory tract may also enhance the ultimate infection of our cells by SARS-CoV-2. This pathway could independently help protect the health and lives of COVID-19 patients if such exogenous cysteine proteases activate the exposure of fusion peptides in protein S, similar to the way they activate hemagglutinin in influenza viruses [43–45]. This information has provided the basis for the development of therapeutic procedures targeting cathepsin L in the respiratory tract to regulate cell infection by the SARS- CoV-2 virus. It is known that after infection with this virus, in order to control COVID-19 disease, the body must acquire antibodies capable of neutralizing these pathogens. This action occurs regardless of which coronavirus variant leads to infection. The role of cathepsin L in airway tissue cells in the "fusion mechanism" is constant and causes activation of protein S regardless of which SARS CoV-2 variant leads to infection. SARS CoV-2 infects. We want to reduce coronavirus fusion by disrupting the control of S-protein activation in SARS-CoV-2, by modifying the enzymes of our body cells to control fusion and the level of threat from COID-19. The premise of our procedure is to obtain antibodies that effectively block a specific pathogen while reducing the risk to patient health and life. Blocking cathepsin L in airway cells should attenuate the activation of the S protein with which SARS-CoV-2 viruses infect, thereby reducing the number of these pathogens in our cells, which should reduce the risk to the health and life of infected patients. "Partial" inhibition of such activation, however, should be sufficient to induce an immune response specific to the infecting virus. We hypothesize that these antibodies are the ultimate therapeutic means of destroying the SARS-CoV-2 virus, including both its known and future variants, in our body. The basis is an invariant cell infection mechanism involving both serine TMPRSS2 and cysteine cathepsin-L, both present in cells of our respiratory system. Our idea is to block only cathepsin-L and only in the human respiratory tract using natural, non-toxic cysteine protease inhibitors that we isolate from natural resources [46].

Discussion and conclusion

Despite the problems with maintaining the activity of acquired antibodies, current COVID-19 vaccines significantly reduce overall morbidity and mortality and are critical to controlling the pandemic. It also appears that individuals who have previously contracted COVID-19 have an enhanced immune response (hybrid immunity) after vaccination. It is suggested that additional exposure to the antigen as a result of "controlled infection" will significantly increase the quantity, quality, and extent of the humoral immune response, regardless of whether it occurred before or after vaccination. The new study found that two forms of immunity - breakthrough infection after vaccination or natural infection after vaccination - provided similar levels of enhanced immune protection. The new approach suggests that it doesn't matter whether someone has a breakthrough infection or has been vaccinated. In both cases, the immune response - measured by serum antibody levels - revealed antibodies that were equally abundant and at least 10 times stronger than the immunity produced by vaccination alone. The likelihood of contracting breakthrough infections is high because there are so many viruses around us these days. However, by getting vaccinated, we put ourselves in a better position. And if we do get infected with a virus, the course of the virus will be milder and we will eventually develop super-resistance. Both groups with "hybrid immunity" achieve higher levels of immunity compared to the group that was vaccinated.

Therefore, we believe that "controlled viral fusion" by blocking cathepsin L in our cells and then acquiring our own antibodies against SARS-CoV-2 may complement post-vaccination immunity [47–49]. Thus, the basic principle of our procedures is not to completely block SARS-CoV-2 fusion, so we do not plan to block serine protease (TMPRSS2), but to limit infection by blocking only cathepsin L so that infecting viruses can elicit a humoral response, that is, the acquisition of active antibodies against a specific infecting pathogen. The level of effective antibodies, which is our goal, will be determined by a serological test. In direct action, we plan to deliver natural cysteine peptidase inhibitors into the respiratory tract and oral cavity via inhalers (aerosols), sprays, lozenges, etc.

Regardless of the direct form, we suggest the possibility of indirect inhalation by inhaling aerosols containing natural cysteine peptidase inhibitors that have been previously delivered there and will then be inhaled by humans in closed rooms. We plan to block cysteine proteases, including both cathepsins and exogenous proteases, from pathogenic microorganisms infecting our airways, thereby reducing the risk of infection and complications of SARS-CoV-2. An example of such microorganisms is the bacterium P. gingivitis, which leads to gingivitis and, when combined with COVID-19, significantly increases the risk of loss of health and life in patients [50,51].

The mechanism of fusion of influenza virus (AH1N1) or other respiratory viruses, including those associated with the common cold, is thought to be similar to SARS-CoV-2 virus activation and fusion in cells. Fusion of these pathogenic viruses begins with the activation of their surface proteins, whether they are S proteins (SARS-CoV-2), influenza virus hemagglutinin (AH1N1), or protein receptors of other viruses. Thus, it can be suggested that blocking cysteine cathepsins (B / L) in the respiratory tract may be used for a wider range of therapies than just SARS-CoV-2 [52–55].

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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