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# Lysosomal proton pump: Its role in SARS-CoV-2's egress and future therapeutic application

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**Abstract.** The coronavirus SARS-CoV-2 is currently spreading throughout the world. The severity of the situation has increased efforts to create efficient prevention and treatment strategies. SARS-CoV-2 has been the subject of numerous experiments, and as a result, scientists now understand it better. Usually, the virus exits the cell through exocytosis. However, SARS-CoV-2 utilizes deacidified lysosomes as a means to egress from the infected cell.This proposal shows a deep understanding of lysosomal viral secretion and elaborates on the impact of the lysosomal proton pump, which functions to regulate the pH value of the endo-lysosomal environment, on lysosome functioning. Experiment are proposed to test the ability of a lysosomal proton pump inhibitor to impede the egress of the SARS-CoV-2 virus and analyze its potential application in a therapeutic target.

**Keywords:** SARS-CoV-2. deacidified lysosomes, lysosomal viral secretion, lysosomal proton pump.

## 1. Introduction

By the end of 2019, the SARS-CoV-2 coronavirus made its initial appearance in Wuhan, China, sparking a global pandemic. The vast majority of enclosed viruses with positive-sense single-stranded RNA genomes are known as coronaviruses. The viruses can be divided into four genera: alpha, beta, gamma, and delta CoVs [1]. Middle East respiratory syndrome (MERS-CoV) and acute respiratory syndrome (SARS-CoV-1) are both caused by previously known human COVs [2]. SARS-CoV-2, a coronavirus, shares structural similarities with SARS-CoV-1 and MERS-CoV. A few years ago, the public was scared by the SARS pandemic. Today, the COVID-19 pandemic has made the phrase "popular" coronavirus once more [3].

SRAS-CoV-2 is now better understood by the general public. Infections with the SARS-CoV-2 virus cause vascular and respiratory diseases. Due to the spike protein (S), which is present at the viral envelope's surface and interacts with the ACE2 receptor to promote viral entry, it falls under the general category of coronaviruses. Positive-sense, single-stranded RNA makes up the SARS-CoV-2 sequence [4]. For structural, non-structural, and accessory proteins, its genome RNA codes. N, M, E, and S proteins are examples of structural proteins. The RNA genome is stabilized by the nucleocapsid protein (N), which packs the genome inside the membrane. Moreover, the membrane contains the membrane protein (M) and the envelope protein (E), which are involved in the organization of the lipid layer that is an essential part of the viral envelope around the virus. Among all the viral proteins,

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the spike protein (S) is the most important part of the virus because it projects from the surface and is the first part of the virus to encounter the host cell [5].

The S protein has two domains: S1 and S2 [6]. The high flexibility of the S1 domain promotes its ability to interact with the ACE2 receptor. The S2 domain, on the other hand, has a cleavage site, which is used for the viral fusion process. Because of the high affinity of the spike protein for the receptor, SARS-CoV-2 has a great ability to infect human cells efficiently. Due to a change on the spike protein, the spike protein enables the virus to enter the cells in different ways, offering the virus the opportunity to spread very fast. The mutation on the spike protein alters the transmissibility of the virus. It either makes the spike protein "sticky" on the host cells' membranes or prevents protective antibodies from binding it. In each way, the virus's infectivity is largely improved [7].

Spike protein can help the viral entrance process in two different ways. The spike protein's S2 domain contributes in one method to the fusion of the viral membrane and the cell plasma membrane. On the outside of respiratory epithelial cells, there is a protease called TMPSS2 (Protease is an enzyme that can cut proteins). S2 is cleaved by the TMPRSS2, mostly resulting in a conformational change in the protein. Because the virus enters these cells first and because they have the ACE2 receptor, the respiratory epithelial cells are always infected at an early stage and patients experience severe lung symptoms. Along with TMPSS2, Furin, on the other hand, may also be in charge of the cleavage of the spike protein [8][9].

For the second way, the virus experiences receptor-mediated endocytosis. In receptor-mediated endocytosis, a virus binds to the cell's specific receptor located at the surface membrane, the ACE2 receptor. When the cathepsin (another protease) responds to the viral spike protein, it lets the membrane folds the virus into endosomes and cleaves it, releasing the virus from the endosome to cytoplasm [10].

The virus's genome begins the cycle of viral replication as soon as it reaches the cell. The viral N proteins fold the genomic RNA into a package once the viral RNA genome is created at the ribosome of the host cell, and then the package budding to the lumen of the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (ERGIC). Later, viral proteins such as membrane protein (M), envelop protein (E), spike protein (S), various non-structural proteins, and auxiliary proteins are integrated with the RNA viral genome. Vesicles would then transport the freshly created virus to the Golgi apparatus and Trans-Golgi Network (TGN) for post-translational modifications [11]. In order to exit the host cell, coronaviruses often use vesicles from the biosynthetic secretory route to merge with the plasma. However, the SARS-CoV-2 coronavirus utilizes a unique lysosomal pathway as a means of egress [12].

Lysosomal proteolytic enzymes only work at specific acidic pH values. The proteolytic enzyme activities are essential for cellular responses, such as cell metabolism, pathogen degradation and antigen presentation. Lysosome acidification is then the prerequisite for lysosomal enzyme stability and enzymatic activity. Even a small increase in pH is enough to block the activities of lysosomal enzymes [13].

Exocytosis is the primary method used by the majority of viruses to egress from cells. In contrast to popular belief, Ghosh (2020) disclosed that the SARS-CoV-2 virus employs lysosomes as its primary mechanism for releasing freshly created viruses last year. It is demonstrated that a decrease in the activity of the lysosomal enzymes was related to the viral egress. Lysosomes in infected cells were deacidified, allowing the virus to go through them without being broken down [14].

The first way to explain the deacidification change is that the lysosomes are loaded with too much cargo and viruses. Another explanation for this is that there may be some perturbations in the lysosomal proton pump or lysosomal membrane ion channel trafficking. Either way, the coronavirus deacidifies lysosomes and utilizes the deacidified lysosomes to transport newly synthesized viruses to the extracellular space via lysosomal trafficking [15].

The V-ATPase proton pump, which is situated in the lysosome membrane, is primarily responsible for the acidic environment of the lysosomes. The flexible membrane-bound proton pump known as vascular-type proton pumping ATPase (V-ATPase) is involved in vesicle trafficking, particularly in

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lysosomes and late endosomes. Proton transport between the cytoplasm and lysosomes is carried out by an enzyme [16]. Unlike other ATPases like F-ATPase and P-ATPase, which use an electrochemical proton gradient to produce ATP, V-ATPase functions normally. Instead, V-ATPase pumps protons to create an electrochemical gradient using the energy from ATP hydrolysis.

V-ATPase is built from the transmembrane $V_0$ and $V_1$ subunits. Both of them have a variety of isoforms that work together to create an enzyme that can transport protons to various locations. Multimeric subunit complexes with a known stoichiometry are $V_1$ and $V_0$. Eight units make up $V_1$ ($A_3$, $B_3$, $C_1$, $D_1$, $E_3$, $F_1$, $G_3$, and $H_1$), but just six make up $V_0$ ($A_1$, $D_1$, E, and the C ring). The catalytic hexamer $A_3B_3$, which consists of three of each A and B subunit, is the component of the V-ATPase that hydrolyzes ATP [17]. The macrolide antibiotics bafilomycin and concanamycin are some selective inhibitors that target the V-ATPase but have no effect on the F-ATPase or P-ATPase. The inhibitors bind to the transmembrane helices of the subunits' interface, which prevents rotation of the subunits.

The protons are transported as a result of the complicated proton pump subunits interacting. In general, $V_1$ is in charge of ATP hydrolysis while $V_0$ is in charge of protons translocation. The ATP energy particle transforms into ADP and releases a phosphate when ATP hydrolysis takes place in the cell on the interface between the A and B subunit (Pi). The central stalk of the V-ATPase, which is made up of subunits D and F of $V_1$, rotates when the Pi is attached to it. The proteolipid ring c then rotates as a result of the central stalk's rotation. Protons then proceed to flow through a channel at a subunit. A glutamate residue on one of the proteolipid subunits (the c ring) must be protonated at subunit a' luminal hemi-channel in order for proton to transport. When the proteolipid ring rotates, the glutamate residue is brought to the cytoplasmic hemi-channel, where it is protonated, and is then able to bind to one side of the hemi-channel. As a result, a positively charged arginine residue in subunit an assists in the release of a proton. From the lysosome to the cytoenvironment, the proton travels via the channel [18].

Surprisingly, according to some research, the V-ATPase is capable of reversible rotational catalysis, which suggests it can utilize an electrochemical proton gradient to synthesize ATP. However, due to the reversed electrochemical proton gradient in endomembrane organelles, V-ATP cannot undertake the reversible process of rotation [19] [20].

While there are many studies about coronavirus entry and replication, there is little known about the pathway for a newly assembled virus to release from the host cells, especially for the mechanism of the lysosomal deacidification. This proposal mainly focuses on the investigation of the function of the lysosomal proton pump in SARS-CoV-2 deacidification and evaluates its future application as therapy in a way of inhibiting the virus' utilization of lysosomes.

## 2. Experiment

### *2.1. Purpose*

The scientific community pays little attention to SARS-CoV-2 egress in comparison to viral entrance and replication processes [21]. Understanding the relevance of the viral egress is crucial, though, as it helps researchers discover efficient patient treatment options [22]. Normally, lysosomes are responsible for the presenting of the pathogen in order for the immune cells to recognize the virus. The deacidification blocks the pathogen presentation process, and hence block the patient's immune response——another good reason for virus to deacidify the lysosysomes. Therefore, it is necessary to investigate the specific way that the virus deacidifies lysosomes. One way that has been proposed is that there may be some interference by the virus of the lysosomal proton pumps which control the H+ pumping. This proposal focuses on designing experiments to test this hypothesis by applying a specific proton pump inhibitor and measuring the lysosomal enzymes' activities and viral egress efficiency to determine whether the proton pump is the mean which the virus utilizes to deacidify the lysosomes.

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### *2.2. Hypothesis*

The V-ATPase proton pump is the means by which the virus deacidifies the lysosome to egress from host cells.

### *2.3. Method and materials*

*2.3.1 Cell Culture.* Primary human tracheal epithelial cells (HTEpC, PromoCell) will be used from surface epithelium of human trachea. Due to their sensitivity, these cells are commonly used to test the function and efficiency of drugs, inhibitors and toxins. Because the bronchus and the trachea are on the pathway of viral infection, HTEpC can imitate the realistic situation of the viral infection, offering scientists useful evidence for therapy design. HTEpC cells are grown in Tracheal Epithelium Growth Medium (Merck Cat No. 511-500) at 37 degrees and 5% $CO_2$ in the humidified incubator. The volume of Tracheal Epithelial Growth Medium is doubled when the culture is >45% confluent [23].

*2.3.2 Treatment with V-ATPase Proton Pump Inhibitor.* Bafilomycin A1* is a V-ATPase inhibitor. It specifically targets the V-ATPase proton pump while it does not affect other ATP proton pumps, such as the F-ATPase proton pump and P-ATPase proton pump. Cells are pre-incubated with exposure to the bafilomycin A1 inhibitor. When the cell culture reaches 45% confluent, 1/100 volume of bafilomycin A1 is dissolved in DMSO* with a final concentration of 50-100 nM, then it is added to the EGM medium (Endothelial Cell Growth Medium) which incubates HTEpC at a temperature of 37°C for one hour in an atmosphere with a $CO_2$ concentration of 5%.

Bafilomycin A1*: Catalog No. S1413, 1mg is bought from Selleckchem website.

DMSO*: Dimethyl Sulfoxide, 500 mL is bought from DMSO-supplier Gaylord Chemical.

The cells samples are divided into two categories: control groups and experiment groups:

**Table 1.** Conditions for experiment group and control group.

| Group Condition | | Infected by the virus | Treated with V-ATPase inhibitor |
|---|---|---|---|
| Control group | Group1 | No | No |
| | Group2 | Yes | No |
| Experiment group | Group3 | No | Yes |
| | Group4 | Yes | Yes |

*2.3.3 Virus Infection.* It is possible to receive the virus from the National Institute of Allergy and Infectious Diseases. It is SARS-CoV-2 isolate USA-WA1/2020 (NIAID). The HTEpC cells in the cultivated HTEpC are infected with the SARS-CoV-2 virus at MOI 1 for 24 hours[17] when the concentration reaches 80% of confluency.

*2.3.4 Measure the Quantitative Viral Egress.* The J1.3 monoclonal anti-M antibody is used (Bosterbio Cat No. M01337) to detect the viral egress. The J1.3 monoclonal anti-M antibody is used to stain the cells after they have been fixed at various post-infection times, including 8 hours, 10 hours, and 12 hours. Anti-M is recognized by a second antibody carrying FITC fluorescence (Selleckchem, Cat No. S6928) to bind to the membrane protein (M) of the newly synthesized virus. Anti-M can recognize the viral M protein on the viral envelope, enabling us to trace the pathway of the virus and observe whether the virus has already left the host cell. As a result, the amount of viral egress is shown by the fluorescence intensity recorded in a Zeiss LSM 880 confocal microscope using the objectives C-

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Apochromat 40/1.2 W and ZEN 2.3 SP1. A graph showing the amount of viral egress is different under the treatment with the proton pump inhibitor at different post-infection times will be shown.

*2.3.5 Measure the Lysosomal Enzyme Activities.* The active sites of the lysosomal enzyme are the target of the lysosome-specific self-quenched substrate (Abcam Cat. No. ab234622), which makes it possible to measure lysosomal enzyme activity. A tiny pH shift indicates deacidification and a decline in enzyme activity. Low background fluorescence is the substrate that is specifically self-quenched by lysosomes. The intensity of substrate fluorescence indicates the intensity of enzyme activities. The self-quenches substrate is a substrate for a lysosomal enzyme. When the enzyme catalyzes the reaction with its substrate, the product fluoresces. The more fluorescence, the higher the enzyme activity. Confocal microscope can measure fluorescent intensity.

*2.3.6 Measure the pH value of Endo-Lysosomal Environment.* A cell-permeable, weak base dye called LysoTracker Red DND-99 (Fisher Scientific, Cat. No. L7528) builds up in lysosomes, late endosomes, and other acidified organelles. Before fixing the cells and staining, the cells are labeled with dye. Cells will be incubated for half an hour with1 mM LysoTracker DND-99 before fixation to detect acidic organelles. Then, fluorescence images can be obtained using either the FV-1000 Confocal Microscope or the Zeiss LSM 880 Confocal Microscope.

Green DND-189 (Fisher scientific, Cat No. L7535) measures a more precise value of pH than the LysoTracker Red DND-99. It has a low pK of 5.2 (the lower the pK value, the stronger the acid). Because it is a strong acid, it requires very acidic environment to fluoresce. Consequently, it doesn't glow outside of very acidic organelles like lysosomes. As a result, its fluorescence amplitude changes precisely with pH.

Uninfected and SARS-Cov2-infected cells can be treated with Lysotracker Red DND-99 (100 nM) and Lysosensor Green DND-189 (1 mM) to detect pH changes. Initially, cells should be scanned using a 458nm Argon laser excitation and 500–550nm bandpass emission filters in order to determine the exact pH value of the acidic organelles. Second, potassium buffers of known pH solutions with 10 M Nigericin antibiotic are used to treat the cells individually. An accurate pH value is obtained by manipulating a standard pH curve after the photos at each pH condition have been captured using the same setup.

*2.4. Expected results*

**Table 2.** Expected results of the experiment.

| pH value (compared with control) | Normal | Very High | Normal | High |
|---|---|---|---|---|
| Measurements Group number | Group1 Uninfected control | Group2 Infected control | Group3 Uninfected in the presence of baflomycin A1 | Group4 Infected in the presence of baflomycin A1 |
| Enzyme activities | Normal | Very low | Normal | Normal |
| Viral egress | No | High rate | No | Low rate |

## 3. Discussion

This experiment result could serve as an indicator to help scientists determine whether an inhibitor targeting the proton pump in lysosomes could be utilized in therapeutic treatment for Covid-19 in the future. It has been previously been suggested that a proton pump inhibitor has some possibilities for use in therapy. However, knowledge at this time is very limited. Therefore, future studies in this field are required. After further research, the role of lysosomal proton pumps in the viral egress process

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could be identified more clearly and comprehensively, and eventually, we can evaluate its feasibility in the therapeutic field.

However, some scientists worry about using inhibition of the lysosomal proton pump as a therapy because they have a significant role in regulating cellular metabolism. The inhibition of the proton pumps may lead to unpredictable damages. In addition, it is necessary to test the specificity of the proton pump inhibitor, truly understanding whether it could handicap proton pumps at other locations of organelles, such as mitochondria [24].

In conclusion, the lysosomes and the role of lysosomal proton pump in viral egress should be noteworthy for scientists, and it deserves further studies. Inhibition of the proton pump may allow control of the virus in patients.

## 4. Conclusion

The results suggests that the SARS-CoV-2 virus modifies the lysosomal V-ATPase proton pump to deacidify lysosomes for its egress, if the data are as expected. The inhibitor that targets the V-ATPase proton pump has a sizable impact on delaying the viral egress process, which could be further investigated and used for the development of therapy options. However, if the results are not as expected, there are some alternatives explanations, for example the lysosomes may be overloaded with too much cargo and too many viruses, or there may be a malfunction of ion channel. Further experiments need to be done to test whether these two hypotheses are correct.

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