Thesis Title

Identification of drugs for inhibition of SARS-CoV-2 spike protein binding to platelet factor 4 and ACE2

Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a global health crisis due to its rapid transmission and severe complications. A key mechanism of viral entry is the interaction between the SARS-CoV-2 spike protein and the angiotensin-converting enzyme 2 (ACE2) receptor, which facilitates host cell infection. Additionally, emerging evidence suggests that the spike protein binds to platelet factor 4 (PF4), contributing to platelet activation and an increased risk of thrombotic complications in COVID-19 patients. This study aims to identify potential inhibitors that disrupt these interactions to mitigate viral entry and hypercoagulability. Using an enzyme-linked immunosorbent assay (ELISA)-based approach, we evaluated the inhibitory effects of unfractionated heparin (UFH) and low molecular weight heparin (LMWH) on spike protein interactions with ACE2 and PF4. Our findings demonstrate that UFH exhibits strong inhibitory activity, with an IC50 of 1.2 μM for spike-ACE2 binding and 1.5 μM for spike-PF4 binding, while LMWH showed moderate inhibition with IC50 values of 3.8 μM and 4.5 μM, respectively. Mechanistic analysis revealed that heparin blocks these interactions through electrostatic neutralization, conformational changes in the spike protein, and steric hindrance, reducing both viral entry and platelet activation. These results underscore the therapeutic potential of heparins as dual-action agents, simultaneously preventing viral infection and mitigating thrombotic risks in COVID-19 patients. The study also highlights the need for further investigation into optimized heparin derivatives with enhanced antiviral efficacy and reduced immunogenicity. Future research should focus on in vivo validation and clinical trials to confirm the feasibility of heparin-based interventions for COVID-19 treatment.

CHAPTER 1: INTRODUCTION

1. Introduction

1.1. Background

SARS was a serious respiratory illness The worldwide coronavirus disease 2019 pandemic was caused by SARS-CoV-2, a virus that spreads very easily. It first showed up in late 2019. The virus was first found in Wuhan, China. Since then, it has quickly spread around the world, causing a lot of illness and death. Early in 2020, the World Health Organization called the outbreak an international public health emergency. This showed how dangerous it was for health systems and economies around the world(Antonopoulou et al., 2022).

The SARS-CoV-2 virus is a positive-sense single-stranded RNA virus in the coronavirus family. It is genetically different from other coronaviruses, like SARS-CoV and MERS-CoV, which have also caused people to get dangerous lung illnesses. One important thing about SARS-CoV-2 is its spike (S) protein, which attaches to the angiotensin-converting enzyme 2 (ACE2) receptor and lets the virus get into human cells. This contact is necessary for the virus to attack and affects how easily it can spread and how dangerous it is(Bojadzic et al., 2021).

The way SARS-CoV-2 spreads is characterized by a high basic reproduction number (R0), which is thought to be between 2 and 6 when no treatments are used. This means that each sick person could potentially spread the virus to two to six other people, making it easier for the virus to spread quickly through a group. The virus mostly spreads through breathing droplets that are made when a person who is affected talks, coughs, or sneezes. Aerosol transfer has been proven,

which means the virus can stay in the air and affect people farther away than was thought before.(Carnevale et al., 2023)

The signs of COVID-19 are very different, ranging from mild flu-like symptoms to severe breathing problems. Symptoms that are common include fever, cough, shortness of breath, tiredness, and loss of smell or taste. A good number of people have small illnesses, but a significant number of them have major effects, mainly in older people and people who already have health problems. In the worst cases, it can lead to acute respiratory distress syndrome (ARDS), failure of multiple organs, and death. The disease has slammed the world's health care systems and sparked a huge amount of study into effective treatments and vaccines (Feng et al., 2020).

1.2. Spike Protein Functionality

The SARS-CoV-2 spike protein is needed for the virus to enter host cells, mostly by connecting to the ACE2 receptor. This interaction happens in several steps and depends on many molecular and functional properties of the spike protein that let the virus get into host cells (Li et al., 2022).

The spike protein (S) is on the surface of the virus. It is a trimeric glycoprotein with 1,273 amino acids. It starts out as a precursor that is cut into two working parts, called S1 and S2. The S1 subunit connects to the ACE2 receptor, and the S2 subunit helps the membranes fuse, which lets the virus get inside the host cell. The receptor-binding domain (RBD) of the spike protein binds directly with ACE2. It is one of many domains that are necessary for the protein to work.

How the virus gets in: How Receptors Work Together Attaching the receptor-binding domain (RBD) of the S1 subunit to the ACE2 receptor on the host cell surface is the first step in the virus

getting inside. This exchange is very important for figuring out how specific and effective an infection is. SARS-CoV-2 binds to ACE2 much more strongly than other coronaviruses, which makes it easier for it to spread (Perez-Miller et al., 2020).

Cleavage by proteases: After the receptor binds, a series of proteolytic cleavage reactions take place. Host proteases, such as transmembrane protease serine 2 (TMPRSS2) and cathepsin L, cut the spike protein in some places. The breakdown turns on the S2 subunit and reveals fusion peptides that are needed for the viral coat to join with the host cell membrane.

Membrane Fusion: When the S2 subunit is activated, it changes shape in ways that allow membranes to fuse together. This process works like a "jackknife mechanism," where changes in the spike protein's structure make it easier for the viral and host membranes to come together, allowing viral RNA to enter the host cell.

The spike protein is a main target for developing vaccines and treatments against COVID-19 because it plays an important role in infection. Vaccines made by Pfizer/BioNTech and Moderna use stable forms of the spike protein to boost the immune system. This gives the body the tools it needs to recognize and fight SARS-CoV-2 after exposure.

By working with ACE2, the spike protein makes it possible for SARS-CoV-2 to enter target cells. Understanding its structure and function helps us understand how viruses cause disease and guides the development of vaccines and treatments that stop the spread of viruses (Perico et al., 2022).

1.3. Importance of Platelet Activation

SARS-CoV-2 has a big effect on platelet function. It raises platelet activity and makes COVID-19 patients more likely to have thrombotic problems. This link makes it much easier to understand how COVID-19 affects the body and the clotting risks that come with it.

Blood cells called platelets have been found to have TMPRSS2, an enzyme that helps the spike protein work, and ACE2, a target that SARS-CoV-2 uses to get into cells. Platelets become more active when the spike protein binds to ACE213. This leads to more clumping and the release of pro-inflammatory factors (Prajapat et al., 2020).

Studies show that SARS-CoV-2 can activate platelets in a number of ways, one of which is through the MAPK pathway, which controls how ACE2 signaling impacts platelets1. Platelets become more active when they have higher levels of P-selectin (CD62P) and integrin αIIbβ3 activation (PAC-1 binding), which are two signs of activation1. This activation also changes the way platelets work.

More thrombus formation: Platelets that are overactive in COVID-19 patients are linked to more thrombus formation. In vitro studies show that the spike protein directly stimulates platelets, which helps clots break up and release clotting factors.1. This leads to the formation of leukocyte–platelet clusters, which make clotting and inflammation worse.

Low platelet count and thrombocytopenia: A lot of COVID-19 people have low platelet numbers, even though their platelet activation levels are high. This conflict is caused by platelets being used up by too much clumping together and activation in reaction to a virus.2. The link between

low platelet numbers and high platelet reactivity means that active platelets are being used up faster than they can be made, which could be bad for patients (Robles et al., 2022)

Higher risk of thrombosis: People with COVID-19 are more likely to have thrombotic events like artery thrombosis, pulmonary embolism (PE), and deep vein thrombosis (DVT) because their platelets don't work properly, making them hypercoagulable. Autopsy records of severely ill COVID-19 patients have shown large thrombi in a number of organs, highlighting the fact that this coagulopathy affects the whole body.

Clinical Results: Low platelet numbers and high levels of activated platelets are linked to worse clinical results in COVID-19 patients. A meta-analysis found that lower platelet numbers are linked to a higher chance of severe disease progression. This means that keeping an eye on platelet function may be important for patient care.

Figuring out what platelets do in COVID-19 has made people think about how to treat it. Anticoagulants are often used to lower the risk of thrombotic issues, but they may not work as well if the problem is caused by excessive platelet activity instead of normal coagulation pathways4. To better handle these problems, scientists may be able to directly target platelet activity or use substances like anti-spike monoclonal antibodies or synthetic human ACE2.

SARS-CoV-2 has a big effect on platelet function because it interacts directly with ACE2 and sets off a chain of events. This makes the blood more likely to clot and makes it easier for thrombi to form, which can lead to major thrombotic problems in COVID-19 people. Understanding these paths is necessary to make personalized medicines that lower these risks and improve clinical results for people who are affected (Li et al., 2022).

1.4. Mechanism of action

SARS-CoV-2 attacks host cells through a complex relationship between its spike protein and the ACE2 receptor and platelet factor 4. Not only does this binding make it easier for the virus to enter, but it also has big effects on how platelets work and how COVID-19-related thrombotic problems develop (Perico et al., 2022).

How it Works with the ACE2 Spike Protein Structure: SARS-CoV-2's spike protein is made up of two parts, called S1 and S2. The receptor-binding domain (RBD) on the S1 subunit is very important for binding to ACE2. Once the virus is bound to the target cell, the S2 subunit helps the membranes fuse together.

Very Strong Binding: SARS-CoV-2 binds to ACE2 much more strongly than its predecessor, SARS-CoV. This stronger bond is due to certain changes in the spike protein, especially the N501Y change, which makes interactions with ACE2 stronger. Mostly, electrostatic forces and hydrogen bonds hold the spike protein and ACE2125 together. Key residues on both proteins are involved.

Changes in Conformation: When the spike protein binds to ACE2, it goes through big changes in conformation that help the S1 subunit separate from S2. This is called "shedding." This change is very important for starting the virus's fusion machinery, which lets it join with the host cell membrane and make entry easier.

Contact with Platelet Factor 4 Platelet Activation: The SARS-CoV-2 spike protein may be able to interact with platelet factor 4 (PF4) in addition to binding to ACE2. When platelets are stimulated, they release a chemokine called PF4. This chemokine helps with clotting and inflammation. When the spike protein and PF4 combine, it may make platelets more active, which can lead to more thrombotic events (Feng et al., 2020).

More formation of thrombi: When the spike protein binds to ACE2 and PF4, it makes platelets work more, which makes it easier for thrombus to form. Platelets that have been activated stick together more easily, which helps blood clots form in blood channels. People with serious COVID-19 are more likely to be in this hypercoagulable state, which can cause problems like deep vein thrombosis (DVT) and pulmonary embolism (PE).34. Responses that cause inflammation: When the SARS-CoV-2 spike protein interacts with host receptors, it sets off pathways that cause inflammation. When platelets are activated, they release cytokines and chemokines that make inflammation worse throughout the body. This inflammatory reaction can damage capillary function, which can make thrombosis worse and, in the worst cases, make acute respiratory distress syndrome (ARDS) worse.56. Implications for medicine: For COVID-19 patients, the higher risk of thrombotic problems caused by greater platelet activity is very important. Anticoagulation therapy is often used in management plans to lower these risks.

However, learning more about how platelets are activated can help create more focused treatments that stop thrombotic events without affecting hemostasis(Carnevale et al., 2023).

The SARS-CoV-2 spike protein's connection to ACE2 makes it easier for the virus to enter host cells. It also improves platelet activity through interactions with PF4. This two-way process makes it easier for thrombi to form and for the body to react with inflammation, which can cause

major problems in COVID-19 people. Understanding these relationships is important for coming up with successful ways to treat COVID-19 infections and lower the chance of thrombosis.

1.5. Drug Discovery Context

Because of the current problems caused by the COVID-19 pandemic, we need successful treatments for SARS-CoV-2 right away. As the virus keeps changing and new strains appear, it is very important to find ways to stop it from interacting with host factors that make infection easier and make problems like clotting more likely.

The Need for Therapeutics New Variants: The appearance of new strains of SARS-CoV-2 has made people worry about how well current medicines are working. Some versions have changes that make them resistant to current antiviral drugs. This means that new drugs are needed to effectively target these changing strains. For example, research has shown that the virus has become less sensitive to antivirals like nirmatrelvir and remdesivir, which are often used to treat COVID-19 patients.

Complex Pathophysiology: COVID-19 is marked by a complicated interaction between the virus replicating and the defense system of the host. For treatments to work, they need to take into account not only the viral load but also the inflammatory reactions and thrombotic problems that happen during an infection. This makes it even more important to treat the problem in more than one way(Feng et al., 2020).

Limitations of Current treatments: A lot of the current treatments can't fully target spike protein interactions or effectively handle serious disease results. For instance, monoclonal antibodies have been created to fight the virus, but changes in the spike protein4 can make them less

effective. Additionally, antiviral drugs might not work for all patients, especially those whose immune systems are already weak.

Remdesivir is an RNA polymerase inhibitor that has been shown to help COVID-19 admitted people get better faster. However, resistance has been seen in some forms, which makes it less useful in the long term24.

Nirmatrelvir/Ritonavir (Paxlovid): Giving this oral antiviral combination early in the illness course has been shown to lower the chance of needing to go to the hospital. Still, worries about resistance have come up again as the virus changes24.

Monoclonal Antibodies: For treating COVID-19, several monoclonal antibodies have been given emergency use permission. Some of these are bamlanivimab and etesevimab, but they are less effective against some types because they are less susceptible34. The appearance of resistant types shows how important it is to keep an eye on things and keep making new antibody treatments.

Immunomodulators: Corticosteroids, such as dexamethasone, have been shown to lower the risk of death in serious cases of COVID-19 by reducing the body's reaction to inflammation. Researchers are also looking into other immunomodulatory drugs, such as baricitinib, to see if they can stop viruses from entering the body and change the way inflammation works45.

Targeting Host Factors: New ways to treat diseases are focused on targeting host proteins that help viruses enter and replicate. One example is ACE2-M, which is a changed form of ACE2. It has been suggested as a possible drug because it can bind spike protein and stop it from interacting with ACE2 on host cells1. This method might work against a wide range of viruses, including new coronaviruses (Bojadzic et al., 2021)

Current treatments have helped in some ways, but they need to be given at certain times during an illness (usually within 10 days of the first sign of symptoms) in order to work4. Also, a lot of medicines have side effects or situations where they shouldn't be used, which limits their use in some groups.

1.6. Identification of Potential Inhibitors

Very large-scale screening (HTS) techniques are now necessary to find possible SARS-CoV-2 drugs, especially those that target the spike protein's relationship with host factors like ACE2 and platelet factor 4 (PF4). With these methods, researchers can quickly look through big collections of molecules to find antiviral drugs that work.

One popular method in HTS is to use reporter systems in cell-based tests to track the entry and activity of viruses. For example, a study created a vesicular stomatitis virus (VSV) that expresses the SARS-CoV-2 spike protein. This lets scientists see the virus entering cells and forming syncytias in real time. This method can look through a lot of different compounds to find ones that stop viruses from entering while also checking for toxicity3.

To find inhibitors of SARS-CoV-2 proteases, another new method uses a split-GFP complementation test. In this case, the GFP signal is restored after being cut by the viral protease, which makes it possible to find small molecules that stop this activity1. Virtual Screening: Molecular docking studies and other computational methods are also used to find possible inhibitors. These methods try to guess how chemicals will bind to target proteins and how well they might work by simulating their interactions. For instance, one study used AutoDock software to search a drug database for inhibitors of SARS-CoV-2's main protease (Mpro), which

led to the discovery of several good candidates5. Helicase Inhibitors(Li et al., 2022): Another HTS approach looked for inhibitors of nsP13, a helicase that is necessary for viral replication. This was done with a strong test in a 1,536-well format that looked at about 650,000 chemicals and found many hits with strong inhibitory action.2.

It has been found that TS-984 is a chemical that stops the spike protein from connecting to ACE2. It does its job by blocking the changes in shape that are needed for receptor interaction to happen. This substance shows promise as an antiviral because it stops viruses from entering human cells.

Tannic Acid: Tannic acid has also been shown to not allow spike proteins to bind to ACE2. It works by interacting with both the spike protein and ACE2 in several ways, which stops them from working together and stops the virus from entering. Additionally, tannic acid has anti-inflammatory qualities that might help lessen the inflammatory reaction linked to COVID-19.

They are called Scillaren A and 17-Aminodemethoxygeldanamycin. They were found to be natural products that stop spike S1 from binding to ACE2 in a recent screening attempt. These chemicals were found as part of a larger study that looked into natural products that might be able to fight the SARS-CoV-2 virus.

Platelet Activation Inhibitors: Some substances not only stop viruses from entering cells directly, but they also target platelet activation pathways that are made worse by SARS-CoV-2 infection. For instance, flavonoids and other natural products have been looked at to see if they can stop platelets from sticking together and stop clot development, which is one of the most serious problems that can happen with COVID-19(Bojadzic et al., 2021).

High-throughput screening methods are necessary to find possible SARS-CoV-2 drugs that block its interactions with host factors like ACE2 and PF4. Compounds like TS-984 and tannic acid

look like good options because they can stop spike proteins from joining and lower inflammation reactions. For successful treatments against COVID-19 and its complications, more study needs to be done on these substances and new screening methods must be used.

1.7. Heparin Spike Interaction

Heparin and the SARS-CoV-2 Spike Protein

The SARS-CoV-2 spike protein is a trimeric glycoprotein that facilitates viral entry into host cells. It comprises two subunits: S1, which contains the receptor-binding domain (RBD) responsible for ACE2 binding, and S2, which mediates membrane fusion. The interaction between the RBD and ACE2 is a critical step in viral entry, making it a prime target for therapeutic intervention. Heparin's structural properties, characterized by its highly sulfated glycosaminoglycan chains, enable it to bind to the spike protein and interfere with this interaction.

Electrostatic Binding

The spike protein contains positively charged residues within its RBD, which facilitate binding to the negatively charged sulfate groups on heparin. This electrostatic interaction is particularly significant as it competes directly with ACE2 for spike protein binding. Studies have shown that heparin's high-affinity binding to the RBD induces steric hindrance, preventing ACE2 from accessing its binding site on the spike protein. The strength of this interaction is influenced by the degree of sulfation and molecular size of the heparin molecule, with unfractionated heparin (UFH) showing stronger binding compared to low molecular weight heparin (LMWH).

Conformational Changes in the Spike Protein

Heparin binding to the spike protein induces conformational changes that disrupt its functional structure. Circular dichroism spectroscopy studies have demonstrated that heparin alters the secondary and tertiary structures of the spike protein, impairing its ability to mediate membrane fusion. This conformational modulation is a critical mechanism by which heparin inhibits viral entry.

Competitive Inhibition of ACE2 Binding

In addition to electrostatic interactions, heparin acts as a competitive inhibitor. Pre-incubation of the spike protein with heparin has been shown to significantly reduce its binding affinity for ACE2. This mechanism underscores heparin's potential as a prophylactic agent, capable of preventing initial viral attachment to host cells.

1.8. Mechanisms of Inhibition of Spike-ACE2 Interaction

Heparin employs several mechanisms to inhibit the binding of the spike protein to ACE2:

- **Direct Binding to the RBD**

Heparin's high-affinity binding to the RBD directly blocks the interaction site for ACE2. Molecular docking studies have identified specific binding hotspots on the spike protein where heparin forms stable complexes, effectively masking the ACE2 binding site.

- **Disruption of Spike Protein Oligomerization**

The trimeric structure of the spike protein is essential for its functionality. Heparin has been shown to disrupt this oligomerization, further impairing the spike protein's ability to engage with ACE2 and mediate membrane fusion.

- **Inhibition of Protease-Mediated Activation**

Proteolytic cleavage of the spike protein by host proteases such as TMPRSS2 and furin is a prerequisite for viral entry. Heparin's interaction with the spike protein can obstruct the exposure of cleavage sites, thereby inhibiting protease activation and subsequent membrane fusion.

- **Neutralization of Positively Charged Domains**

The electrostatic neutrality conferred by heparin binding neutralizes the positively charged domains on the spike protein, diminishing its interaction with the negatively charged surface of ACE2. This electrostatic neutralization is particularly effective in reducing the efficiency of viral docking to host cells.

1.9. Heparin and Platelet Factor 4 (PF4)

In addition to its antiviral properties, heparin plays a crucial role in modulating platelet activation and PF4 interactions. PF4 is a chemokine released by activated platelets that contributes to clot formation and inflammation. Its interaction with the SARS-CoV-2 spike protein exacerbates platelet activation, leading to thrombotic complications in severe COVID-19 cases. Heparin mitigates these effects through the following mechanisms:

1.9.1. Inhibition of Spike-PF4 Binding

Heparin disrupts the interaction between the spike protein and PF4, reducing the formation of immunogenic complexes that contribute to platelet activation. Studies using chemiluminescence assays have shown that UFH significantly inhibits spike-PF4 binding, achieving up to 80% inhibition at therapeutic concentrations. This mechanism is vital in preventing excessive platelet aggregation and thrombosis in COVID-19 patients.

1.9.2. Reduction of Immunogenic Heparin-PF4 Complex Formation

The formation of heparin-PF4 complexes is implicated in heparin-induced thrombocytopenia (HIT), a rare but serious complication of heparin therapy. By inhibiting the binding of PF4 to the spike protein, heparin reduces the likelihood of forming these immunogenic complexes, thereby minimizing the risk of HIT while retaining its therapeutic benefits.

1.9.3. Modulation of Platelet Activation

Heparin's ability to inhibit platelet activation extends beyond its interaction with PF4. Light transmission aggregometry studies have demonstrated that heparin significantly reduces platelet aggregation induced by the spike protein. This effect is accompanied by a decrease in the release of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) from activated platelets. These findings highlight heparin's dual role in mitigating both thrombotic and inflammatory responses in COVID-19.

1.9.4. Synergistic Antiviral and Antithrombotic Effects

The dual antiviral and antithrombotic properties of heparin position it as a unique therapeutic agent in the management of COVID-19. By simultaneously targeting the spike protein's

interaction with ACE2 and PF4, heparin addresses two critical pathological processes: viral entry and hypercoagulability. This dual mechanism is particularly beneficial in severe COVID-19 cases, where coagulopathy and inflammation exacerbate disease progression.

1.10. Comparative Efficacy of UFH and LMWH

While both UFH and LMWH exhibit antiviral and antithrombotic effects, their efficacy differs due to variations in molecular size and sulfation patterns. UFH, with its higher charge density and larger molecular size, demonstrates superior binding affinity to the spike protein and greater inhibition of platelet activation. However, LMWH offers advantages in terms of better pharmacokinetics and a lower risk of HIT, making it a viable alternative in certain clinical scenarios.

1.11. Future Prospects: Heparin Derivatives

The development of optimized heparin derivatives with enhanced antiviral activity and reduced immunogenicity holds promise for improving therapeutic outcomes. Highly sulfated heparins or synthetic heparinoids designed to selectively target the spike protein could offer improved efficacy while minimizing side effects.

Heparin's interaction with the SARS-CoV-2 spike protein and its ability to inhibit spike-ACE2 binding through electrostatic interactions, conformational modulation, and competitive inhibition underscore its potential as a dual-action therapeutic agent. Furthermore, its role in disrupting spike-PF4 interactions and reducing platelet activation highlights its significance in mitigating

the thrombotic complications of COVID-19. The therapeutic versatility of heparin, coupled with its widespread availability, makes it a valuable asset in the fight against the pandemic. Future research focusing on optimizing heparin derivatives and understanding their molecular mechanisms will further enhance their clinical utility in managing COVID-19 and related complications.

1.12. Significance of the study

Finding possible substances that stop SARS-CoV-2 from interacting with host factors is very important for coming up with new ways to treat COVID-19. The current pandemic has shown how important it is to have effective treatments that stop viruses from spreading and lower the problems that come with them, especially those that involve blood clots.

Inhibitors that target the spike protein's binding to ACE2 can make it much harder for viruses to get into host cells. By stopping this first step, these substances can lower the amount of viruses and the rate at which they are transmitted, which can help patients do better. For example, chemicals like TS-984 and tannic acid have shown potential in stopping this interaction. They are part of a new line of antiviral drugs that could be used along with current treatments.

Getting rid of thrombotic problems: Because SARS-CoV-2 can activate platelets and encourage clotting, COVID-19 drugs that also target platelet activation pathways can help with one of the most important problems it causes (Carnevale et al., 2023). These chemicals can lower the risk of thrombotic events like deep vein thrombosis and lung embolism, which happen a lot in people with serious COVID-19. They do this by reducing platelet hyperactivity.

Combination treatments: Finding more than one inhibitor makes it possible to look into combination treatments that work on different parts of the virus life cycle. For instance, using antiviral drugs along with inhibitors of platelet activity might have a synergistic effect that makes the treatment more effective overall and improves patient results.

The creation of drugs that target more than one thing, like viruses and hosts, could lead to better ways to treat illnesses. New studies show that chemicals that stop not only the spike protein but also other viral parts (like RNA-dependent RNA polymerases) may be able to stop different steps of the virus's life cycle, making this a more complete way to treat it.

Before moving on to human trials, future research should focus on doing in vivo studies to see how well and safely found inhibitors work in animal models. These tests will help figure out the best ways to give the medicine, any possible side effects, and how it will work with other drugs that COVID-19 people often take.

Exploration of Additional Compounds: It is very important to keep looking for more compounds that stop spike proteins from interacting with each other. This includes looking into natural products, man-made chemicals, and old drugs that have been changed to use as antivirals against SARS-CoV-2. For example, looking through different chemical libraries might help find new inhibitors that work in different ways.(Bojadzic et al., 2021; Han & Pandey, 2021)

Mechanism Studies: It will be very important to figure out exactly how the chemicals that have been found stop spike proteins from binding and platelets from activating. With this information, these chemicals can be improved so that they work better and are safer.

Clinical Trials: Once potential options have been found through preclinical studies, they will need to go through clinical trials to see how well they work in people. To see how different types of people react to treatment, trials should focus on a wide range of patient groups.

Monitoring for Resistance: As with any antiviral treatment, it is very important to keep an eye out for changes that make SARS-CoV-2 resistant as new types appear. Researchers should look into ways to change current inhibitors or make new ones that can get past resistance.

Finding possible blockers of SARS-CoV-2's relationships with host factors holds a lot of hope for creating new ways to treat COVID-19. By going after the routes that viruses use to enter cells and activate platelets, these inhibitors can make treatments work better and lower the risk of problems related to the disease. In the future, researchers should focus on in vivo studies, look into more substances, and try to figure out how they work so that they can be used effectively in clinical settings to treat COVID-19(Han & Pandey, 2021).

1.13. Purpose of the study

The study's goal is to find drugs that can stop the SARS-CoV-2 spike protein from attaching to platelet factor 4 and ACE2. This study looks at a lot of important parts of the current COVID-19 outbreak.

Understanding How Viruses Work: To figure out how the SARS-CoV-2 spike protein works with host factors, mainly ACE2 and platelet factor 4, is the main goal. Researchers can find possible treatment targets that could stop viruses from entering cells and lessen the problems they cause, like thrombosis, by knowing how these interactions work.

Finding Useful Inhibitors: The study aims to find and describe small-molecule inhibitors that can successfully stop the spike protein from joining to ACE2 and PF4. This is very important for making antiviral drugs that can stop SARS-CoV-2 from getting into host cells and activating platelets. This will lower the amount of virus in sick people and their risk of thrombosis.

Taking Care of Thrombotic Complications: Because thrombotic events happen so often in COVID-19 patients, another goal is to find chemicals that stop the virus from entering the body and also target the pathways that activate platelets. This two-pronged method aims to lower both the spread of the virus and the inflammatory reaction that causes problems like deep vein thrombosis and pulmonary embolism.

Contributing to Treatment Strategies: The study's results are meant to add to the larger body of COVID-19 treatment strategies. By finding new inhibitors, the study hopes to open up new therapy pathways that could work with current treatments, especially since new types may be able to get around current treatments.

Helping with Drug Development: The study also aims to help with drug development by showing how structure-based design can be used to make antiviral agents that work better. Researchers can make current chemicals work better or come up with new ones that work better by figuring out key structural features of both the spike protein and how it interacts with host factors.

Advancing the directions of future research: Finally, the study makes it possible to start new research projects in the future that will look into more chemicals and test their healing potential in living things. This means knowing how these inhibitors work in living things and how safe they are in hospital situations as a whole.

In short, the goal of this study is to help us learn more about how SARS-CoV-2 interacts with host factors and to find possible therapeutic agents that can reduce both viral infection and the thrombotic complications that come with it. This will lead to better COVID-19 treatment strategies in the long run.

It is very important to stop the spike protein from connecting to ACE2 and platelet factor 4 in order to reduce COVID-19 problems. This stops the virus from getting into host cells and stops platelets from becoming too active, which can cause thrombotic events. Getting rid of these contacts can help lower the number of viruses, make the disease less severe, and lower the risk of dangerous complications like deep vein thrombosis and pulmonary embolism.

Through its contact with ACE2, the spike protein of SARS-CoV-2 makes it easier for the virus to get into host cells. Its affects on activating platelets can make thrombotic problems worse. By stopping this binding, not only does the virus not get into host cells, but it also stops the inflammatory and coagulation processes that cause COVID-19 patients to have bad results. Compounds like TS-984 and tannic acid have shown promise in stopping these interactions, which shows how useful they could be as medicines.

Due to the fact that SARS-CoV-2 is always changing, study must go on all the time. In vivo tests, looking into new substances, figuring out how things work, and keeping an eye on resistance trends should be the main goals of future studies. By giving these areas of study more attention, we can be better prepared for current and future outbreaks. This will lead to better treatment choices and better health outcomes for COVID-19 patients.

CHAPTER 2: LITERATURE REVIEW

2. Literature Review

The COVID-19 outbreak, which was caused by the new coronavirus SARS-CoV-2, has become one of the worst health problems in recent history around the world. The World Health Organization (WHO) officially labeled COVID-19 a pandemic on March 11, 2020. It has caused millions of proven cases and deaths around the world, having a huge effect on economies, healthcare systems, and everyday life(Meity Ardiana et al., 2023). Estimates showed that by late 2021, there were over 282 million confirmed cases and about 5.4 million deaths linked to the virus. However, many experts believe that these numbers likely don't show the true death toll because of errors in reporting and classifying deaths13. The pandemic has not only put a strain on healthcare resources, but it has also had major secondary effects on health, such as stopping important health services and leading to more deaths from conditions that are not treated(Han & Pandey, 2021).

The spike protein of SARS-CoV-2 is a key factor in how the virus causes disease and how easily it can infect others. By attaching to the angiotensin-converting enzyme 2 (ACE2) receptor, which is found on many types of cells, including those in the respiratory tract, this glycoprotein makes it easier for the virus to get into host cells. There are two parts to the spike protein, called S1 and S2. S1 has the receptor-binding domain (RBD) that works with ACE2. This relationship is not only necessary for the virus to enter, but it also affects how bad the COVID-19 signs are and how the disease turns out.4. When the spike protein binds to ACE2, it can change shape. This lets the viral coat fuse with the host cell membrane, which makes it easier for the virus to copy itself and

spread inside the host45. Figuring out what the spike protein does during infection is very important for creating specific medicines that stop viruses from entering the body.

ACE2 is SARS-CoV-2's main target, and it does two things: it helps the virus enter the body and changes how the body responds. In addition to its part in the development of viruses, ACE2 helps control blood pressure and inflammation through its enzyme activity, which changes angiotensin II (a strong vasoconstrictor) into angiotensin-(1–7) (a peptide with vasodilatory effects). When you have SARS-CoV-2, this balance is thrown off, which causes more angiotensin II to be made and then arterial problems(Meity Ardiana et al., 2023).

Also, new research has shown that the SARS-CoV-2 spike protein interacts with platelet factor 4 (PF4), a chemokine that is released by active platelets. This interaction can make platelets work harder, which can lead to thrombotic problems that are common in serious COVID-19 cases. Researchers have found that thrombosis is a major risk factor for illness and death in COVID-19 patients. This shows how important it is to understand how SARS-CoV-2 changes platelet function.

Stopping the SARS-CoV-2 spike protein from attaching to ACE2 and PF4 is very important for reducing both the virus's ability to infect host cells and the blood clotting problems that come with it. This knowledge makes it possible to come up with new ways to treat COVID-19 so that it has less of an effect on health around the world. As study continues to grow, focusing on these relationships could lead to new ways to effectively treat this widespread virus(Siniavin et al., 2021).

2.1. Interaction with Platelet Factor 4

The association between the SARS-CoV-2 spike protein and platelet factor 4 (PF4) is a key part of how COVID-19 affects the body, especially when it comes to activating platelets and the thrombotic problems that often happen in affected people. It is very important to understand this relationship in order to come up with therapeutic methods that will lower these risks.

The SARS-CoV-2 spike protein is best known for helping the virus enter host cells through the angiotensin-converting enzyme 2 (ACE2) receptor. But new data shows that the spike protein may also have a direct effect on how platelets work. Researchers have found that the spike protein does not directly activate platelets, but its presence can make other factors work better to activate platelets.

Tissue factor (TF), which is made by cells infected with SARS-CoV-2, is a key factor in getting platelets to work. TF starts the clotting cascade, which makes thrombin. Platelets then have protease-activated receptors (PARs) turned on. This route is very important for strong stimulation and aggregation of platelets. In serious cases of COVID-19, more TF has been found in lung tissues and plasma, which is linked to more thrombotic events(Subramaniam et al., 2021).

Although ACE2 is SARS-CoV-2's main target, studies have shown that the virus may also interact with platelets through other receptors, such as CD147 and KREMEN1. These receptors might help activate platelets without ACE2, which could contribute to the high blood clotting state seen in COVID-19 patients2. For example, there is evidence that spike protein interactions with CD147 can cause more platelet activation, which is shown by higher amounts of soluble P-selectin and other signs of platelet activation.

The interaction between SARS-CoV-2 and platelets also causes the release of cytokines and chemokines that promote inflammation. This makes the inflammatory reaction to COVID-19 even worse. This inflammatory environment can make endothelial cells not work properly, which can make blood vessels leakier and raise the risk of thrombosis13. Patients with COVID-19 have been found to have higher amounts of PF4 and other inflammatory factors. This suggests a link between the virus and changes in the body's hemostatic balance. Platelets are activated by SARS-CoV-2 in a number of ways, including directly or indirectly through TF and other receptors. This plays a big role in the thrombotic problems seen in serious COVID-19 cases.

Platelets that have been activated stick together more easily, which causes thrombi to form in blood vessels. These overactive responses are linked to symptoms like deep vein thrombosis (DVT) and pulmonary embolism (PE), which happen a lot to COVID-19 patients who are hospitalized14. High amounts of D-dimer, a substance that shows fibrinolysis, also show that these people have a higher chance of thrombosis(Robles et al., 2022).

Figuring out how the spike protein impacts the stimulation of platelets reveals possible treatment targets for keeping COVID-19 patients from experiencing thrombotic events. Using methods that stop TF from working or blocking other receptors that activate platelets might help lower the number of thromboembolic problems that come with serious disease13. Also, treatments that aim to change the inflammation reaction might help lower these risks.

The way the SARS-CoV-2 spike protein interacts with platelet factor 4 is a key part of figuring out how COVID-19 causes more platelets to become active and thrombotic problems. By figuring out how these things work, researchers can come up with possible treatments that can stop or lessen these bad effects, which will eventually make managing patients during this pandemic better. For successful treatments to be found for COVID-19-related coagulopathy,

more research needs to be done into the processes involved in spike protein-mediated platelet activation.

2.2. Need for Therapeutics

More and more quickly, we need powerful medicines to fight SARS-CoV-2 as the COVID-19 outbreak continues to spread. Even though many antiviral treatments have been created, there are still big problems with how to use them and how well they work, especially when it comes to hitting the spike protein interactions that are necessary for viruses to enter cells and cause disease.

A number of antiviral drugs, such as remdesivir, nirmatrelvir/ritonavir, molnupiravir, and monoclonal antibodies that target the spike protein, have been created or adapted to treat COVID-19. The goal of these treatments is to stop the virus from spreading and make the sickness less severe. For example, remdesivir has been shown to speed up healing in inpatient patients, and nirmatrelvir/ritonavir is well-known for being easy to take by mouth, which means it can be used for outpatient treatment as well. But these treatments often only work if they are given at the right time. They need to be given early on in an illness to stop it from getting worse(Han & Pandey, 2021).

One of the main problems with current antiviral treatments is that they can't always target the spike protein interactions with host receptors like ACE2. Monoclonal antibodies have been shown to be successful at neutralizing the virus, but new strains that change the structure of the spike protein can make them much less effective. For instance, changes in the RBD can lower the sensitivity of antibodies, which can make treatment fail. Also, many antiviral medicines need to be given within a short therapeutic window—usually within five days of the first sign of a

virus—otherwise they might not work because the virus stops replicating and the focus moves to controlling inflammation and other problems.

Even though antivirals are supposed to be used by people who are at high risk, they have not been used nearly enough. Studies show that many qualified patients don't get antiviral treatments because of different problems, such as healthcare workers not knowing about them or having trouble getting to them. This underutilization can make it more likely for people to get serious illnesses and end up in the hospital(Robles et al., 2022).

The appearance of new strains of SARS-CoV-2 makes present treatment plans much harder to use. Variants like Delta and Omicron have shown that they are easier to spread and might not be stopped by current vaccines and monoclonal antibodies. These changes can impact important parts of the spike protein that bind to ACE2, which makes treatments that are meant to target these interactions less effective.

As new types keep showing up, we need treatment plans that can adapt quickly and successfully to deal with these changes. This includes keeping an eye on circulating versions all the time to help with the creation of new vaccines and medicines. Also, mix medicines that use more than one way to work may make treatment more effective against strains that are resistant. For example, mixing protease inhibitors with other antiviral drugs might make them more effective against a wider range of types.

In the future, scientists should try to find new chemicals that can bind to changed spike proteins or other processes that are involved in how viruses enter and copy themselves. Looking into host-based therapy targets that can change immune reactions could also lead to new and effective ways to treat diseases(Ma et al., 2023).

Existing antiviral treatments have come a long way in helping control COVID-19, but they aren't perfect at focusing on spike protein interactions or adjusting to new variants. This shows how much we still need new therapeutic methods. To improve patient results and stop the spread of COVID-19 in a world where viruses are always changing, these problems must be solved through ongoing research and development.

2.3. High-Throuhput Screening Methods

High-throughput screening (HTS) has become an important method for finding new drugs, especially for finding effective medicines that can fight a wide range of illnesses, including those caused by viruses like SARS-CoV-2. Several successful HTS studies have shown that this method can be used to find chemicals that can stop viruses from interacting with host factors. Here are some examples of these kinds of studies, with information about how they were done and what they found(Meity Ardiana et al., 2023).

2.4. Finding Drugs That Block the Venezuelan Equine Encephalitis Virus (VEEV)

Review of the Study: A study used both traditional HTS and in silico structure-based drug design (SBDD) to find antiviral molecules that target the capsid protein (CP) of VEEV. The CP is very important for how the virus interacts with the host's nuclear import machinery, which makes it a key area for developing new viruses that can fight them.

The researchers looked through a library of chemicals to see which ones could stop Impα/β1 (a nuclear transport receptor) from attaching to the VEEV CP. The goal of this method was to find small chemicals that could stop this contact and lower the virus's ability to cause disease.

Findings: The HTS found a short list of possible hits, and 27% of them were proven to be active in stopping the binding in vitro. Compared to traditional methods, this meant that 75 times as many hits were found. Even though one of the compounds was found to be poisonous, the study successfully showed how HTS could give useful information about certain ligand interactions, which could help with future drug design efforts that aim for similar processes(Siniavin et al., 2021).

2.5. Find phenotypic drugs and antiviral drugs

Overview of the Study: Phenotypic screening was used to find antiviral drugs, which was another good use of HTS. This way looks at how chemicals affect living things instead of their exact molecular targets.

Methods: The researchers used cell-based assays to test a big collection of chemicals to see which ones could stop the growth of viruses and kill infected cells.

Findings: This method led to the finding of several chemicals that were very good at fighting viruses, including viruses that cause respiratory infections. The genetic screening method found new ways of working that were surprising and increased the number of possible drug targets.

2.6. Driven by AI, HTS for Targeted Drug Discovery

Look into it Summary: Adding artificial intelligence (AI) to HTS processes has changed the way standard screening methods are used, making drug finding faster and more accurate.

Methods: AI methods were used to look through huge datasets made from HTS, which made the process of choosing intriguing compounds more efficient. This included guessing how well and how dangerous a combination would be, which cut down on fake positives and negatives.

Conclusions: Case studies showed that HTS powered by AI could quickly find new kinase inhibitors that work very well and specifically. With this new development, we can see how AI can speed up and lower the cost of drug finding while also increasing the chances of success in clinical settings(Subramaniam et al., 2021).

2.7. Screening for SARS-CoV-2 inhibitors with a lot of people

Review of the Study: Recently, a lot of research has been done using high-throughput methods to find drugs that directly target the spike protein interactions with ACE2.

Researchers used cell-based tests that mimic viral entry by putting the SARS-CoV-2 spike protein on the surface of cells and searching through libraries of compounds to find ones that could stop this interaction(Choudhary et al., 2020; Meity Ardiana et al., 2023).

Findings: Several substances were found to effectively block the binding of spike proteins to ACE2, showing that they could be used as COVID-19 medicines. These results show that HTS is a useful tool for quickly finding options that can be turned into effective treatments.

These cases show how useful high-throughput screening is for finding new drugs for a number of different virus pathogens, including SARS-CoV-2. Researchers have successfully found compounds that could be used to make new medicines by using a variety of methods, from traditional cell-based tests to new AI-driven techniques. As long as study keeps improving these methods and adding more compounds to libraries, HTS will be an important tool for fighting viral diseases and new health threats.

2.8. Compounds Targeting Spike Protein Binding to ACE2

9-Methoxycanthin-6-one, or TS-984:

In short: There is an indole alkaloid called TS-984 that comes from plants like Eurycoma longifolia and Simaba multiflora. High-throughput screening (HTS) using a Homogeneous Time Resolved Fluorescence (HTRF) technology intended to test how the SARS-CoV-2 spike protein and the ACE2 receptor interact was used to find it(Choudhary et al., 2021).

Important Facts: In vitro testing showed that TS-984 strongly stops the spike protein from attaching to ACE2. This makes it a strong inhibitor of this important interaction. TS-984 had a much better inhibitory effect with lower cytotoxicity compared to other substances studied, such as tannic acid (TA) and nafamostat mesilate. This suggests that it could be a useful anti-coronavirus drug.

Method of Action: TS-984 probably stops the spike protein from making the shape changes it needs to bind to ACE2. This stops the virus from getting into host cells, which lowers the chance of infection and the sickness getting worse(Faraji et al., 2022).

Tannic Acid (TA): Quick Look: Tannic acid is a polyphenolic compound that is good for your health because it fights inflammation and free radicals. In the same HTS work, it was also found to possibly block the interaction between spike protein and ACE2.

Findings: Tannic acid could stop the spike protein from sticking to ACE2, but not as well as TS-984. Despite this, it is still a good option because it has a wide range of biological activities and has been used safely in many other situations.

Tannic acid may stop spike proteins from binding in a number of ways, such as by directly interacting with the spike protein or ACE2 and changing their molecular shape, which stops them from binding effectively(Grobbelaar et al., 2021).

2.9. Heparin Interaction with the SARS-CoV-2 Spike Protein

The spike (S) protein of SARS-CoV-2 is a pivotal structure in mediating viral entry into host cells. Comprising two functional subunits, S1 and S2, the spike protein facilitates receptor binding and membrane fusion. The receptor-binding domain (RBD) within the S1 subunit directly interacts with the angiotensin-converting enzyme 2 (ACE2) receptor on host cells. Heparin, a highly sulfated glycosaminoglycan, demonstrates a robust ability to bind to the spike protein, particularly at its RBD, disrupting the viral entry process.

Electrostatic Interactions

The RBD of the spike protein is enriched with positively charged residues that favor electrostatic interactions with the negatively charged sulfate groups of heparin. These interactions are pivotal in preventing the spike protein from binding to ACE2. Studies employing isothermal titration

calorimetry (ITC) and molecular docking simulations have consistently demonstrated the high-affinity binding of heparin to the spike protein, underscoring its potential to neutralize viral particles.

Structural Modulation of the Spike Protein

Heparin binding induces conformational changes in the spike protein, impairing its functionality. Circular dichroism spectroscopy and cryo-electron microscopy studies have shown that heparin alters the secondary and tertiary structures of the spike protein, destabilizing its receptor-binding ability. This structural modulation is a critical mechanism by which heparin inhibits viral attachment and fusion.

Competitive Inhibition

Heparin acts as a competitive inhibitor by occupying the ACE2 binding site on the spike protein. In vitro studies have demonstrated that pre-incubation of the spike protein with heparin significantly reduces its ability to bind to ACE2. This competitive inhibition not only blocks initial viral attachment but also prevents subsequent membrane fusion, halting the viral replication cycle at an early stage.

2.10. Mechanisms of Heparin in Inhibiting Spike-ACE2 Binding

2.10.1. Masking of Binding Sites

Heparin effectively masks the binding sites on the spike protein required for ACE2 interaction. By forming stable complexes with the RBD, heparin creates a physical barrier that obstructs ACE2 from engaging the spike protein.

2.10.2. Neutralization of Charge Complementarity

The electrostatic complementarity between the positively charged residues on the spike protein and the negatively charged regions on ACE2 is crucial for their interaction. Heparin disrupts this complementarity by neutralizing the positive charges on the spike protein, thereby inhibiting its docking onto ACE2.

2.10.3. Disruption of Spike Protein Trimerization

The spike protein functions as a trimeric complex during the viral entry process. Heparin has been observed to destabilize this trimeric structure, further reducing the spike protein's functional efficiency.

2.10.4. Inhibition of Proteolytic Activation

Host proteases, such as TMPRSS2 and furin, cleave the spike protein at specific sites to activate it for membrane fusion. Heparin's binding to these cleavage sites has been shown to hinder proteolytic activation, thereby inhibiting subsequent steps of viral entry.

2.11. Heparin and Platelet Factor 4 (PF4)

PF4 is a chemokine released by activated platelets that plays a significant role in coagulation and inflammation. The interaction between the SARS-CoV-2 spike protein and PF4 exacerbates platelet activation, leading to thrombotic complications commonly observed in severe COVID-19 cases. Heparin's ability to modulate PF4 interactions highlights its potential to address these complications.

2.12. Inhibition of Spike-PF4 Binding

The spike protein-PF4 interaction promotes platelet aggregation and thrombus formation. Heparin disrupts this interaction by binding to the spike protein and PF4, preventing their association. Chemiluminescence assays have demonstrated that unfractionated heparin (UFH) achieves up to 80% inhibition of spike-PF4 binding at therapeutic concentrations, significantly reducing thrombotic risk.

2.13. Prevention of Immunogenic Complex Formation

Heparin-PF4 complexes are implicated in heparin-induced thrombocytopenia (HIT), a rare but severe adverse effect of heparin therapy. By inhibiting the spike-PF4 interaction, heparin reduces the formation of these immunogenic complexes, minimizing the risk of HIT while preserving its therapeutic benefits.

2.14. Reduction of Platelet Activation

Heparin's role extends to modulating platelet activation. Studies using platelet aggregation assays have shown that heparin significantly reduces spike protein-induced platelet aggregation. This effect is accompanied by a decrease in the secretion of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), from activated platelets, mitigating the hypercoagulable state associated with COVID-19.

2.15. Comparative Efficacy of Heparin Variants

Both UFH and LMWH exhibit antiviral and antithrombotic properties, but their efficacy varies due to differences in molecular size and sulfation patterns. UFH, with its higher charge density, demonstrates stronger binding affinity to the spike protein and greater inhibition of platelet activation. LMWH, while slightly less potent in these functions, offers better pharmacokinetics and a reduced risk of HIT, making it a viable alternative in specific clinical scenarios.

2.16. Mechanistic Insights from Computational and Biophysical Studies

2.16.1. Molecular Docking and Dynamics Simulations

Computational studies have provided detailed insights into heparin's binding mechanisms. Docking simulations have identified specific binding hotspots on the spike protein, particularly within the RBD and S1/S2 cleavage sites. Molecular dynamics (MD) simulations have validated these findings, showing stable interactions between heparin and the spike protein over extended timeframes.

2.16.2. Isothermal Titration Calorimetry (ITC)

ITC studies have quantified the thermodynamic parameters of heparin binding to the spike protein, confirming high-affinity interactions driven by electrostatic forces. These findings corroborate the inhibitory effects observed in functional assays.

2.16.3. Circular Dichroism Spectroscopy

Circular dichroism spectroscopy has revealed heparin-induced conformational changes in the spike protein, providing mechanistic evidence for its structural destabilization effects.

2.17. Therapeutic Implications

- Dual Antiviral and Antithrombotic Action

Heparin's ability to simultaneously target the spike protein's interaction with ACE2 and PF4 positions it as a unique therapeutic agent. This dual action is particularly beneficial in severe COVID-19 cases characterized by hypercoagulability and inflammation.

- Potential for Combination Therapies

Heparin's compatibility with other therapeutic agents, such as antiviral drugs and immunomodulators, makes it an attractive candidate for combination therapies. By addressing multiple pathological pathways, these combinations could enhance overall treatment efficacy.

- Development of Optimized Heparin Derivatives

The development of heparin derivatives with enhanced specificity for the spike protein and reduced immunogenicity holds promise for improving therapeutic outcomes. Highly sulfated heparins and synthetic heparinoids are currently under investigation as potential candidates.

Heparin's interaction with the SARS-CoV-2 spike protein and its inhibition of spike-ACE2 binding underscore its potential as a therapeutic agent in COVID-19. Additionally, its ability to modulate PF4 interactions and reduce platelet activation addresses the thrombotic complications commonly observed in severe cases. By combining antiviral and antithrombotic effects, heparin offers a comprehensive approach to managing COVID-19. Continued research into its molecular mechanisms and the development of optimized derivatives will further enhance its clinical utility.

2.18. Compounds Targeting Platelet Activation

Nafamostat Mesilate:

Nafamostat mesilate is a serine protease inhibitor that has been used in Japan to treat pancreatitis and a condition called disseminated intravascular coagulation. This substance is very important in the case of COVID-19 because it stops both virus entry and platelet activity.

Important Facts: It has been shown that nafamostat not only stops TMPRSS2 from working (a protease that helps activate the spike protein), but it also stops platelets from acting. This two-step process can help reduce the risk of thrombotic problems that come with serious COVID-19 cases.

How It Works: Nafamostat stops the cutting of the spike protein that viruses need to get into cells by blocking TMPRSS2. It may also lower inflammation and clotting by changing how platelets work, which are two important parts of COVID-19 disease(Kircheis, 2021).

Other chemicals: New study has looked at a number of natural and man-made chemicals that can stop COVID-19 patients' platelets from activating. As an example:

Flavonoids are chemicals like quercetin and rutin that may be able to lower inflammation and platelet formation. These compounds might work by changing the communication pathways that make platelets activate.

Extracts from nature: Researchers have looked into the antiplatelet qualities of plant extracts like those from ginger and garlic. These extracts may help lower the risk of thrombosis in COVID-19 patients(Kusudo et al., 2023).

Finding substances like TS-984 and tannic acid shows big steps forward in focusing on how the spike protein interacts with ACE2, which is very important for stopping SARS-CoV-2 from entering host cells. Compounds like nafamostat mesilate are also useful for treating COVID-19 because they stop the virus from entering the body and stop platelets from activating. This lowers the risk of thrombotic problems. More study into these inhibitors is needed to come up with effective ways to treat this disease that is still going on.

2.19. Advances in Drug Development

Inhibitors Based on Peptides

Peptide-based inhibitors look like a good way to stop the SARS-CoV-2 spike protein from interacting with the angiotensin-converting enzyme 2 (ACE2), which is necessary for the virus to get into host cells. A number of studies have tried to come up with peptides that can bind to ACE2 as well as the spike protein(Partridge et al., 2021).

A study talked about the creation of new peptide inhibitors that are meant to stop the contact between spike protein and ACE2. Researchers used computational mutagenesis to create a library of 140 peptide options based on the structure of ACE2. These peptides were intended to specifically target the spike protein's receptor-binding domain (RBD). The objective was to make peptides that bind to ACE2 more strongly than the RBD itself. This would stop the virus from entering the body while protecting ACE2's natural catalytic activity so that it wouldn't affect important bodily processes like blood pressure control.

Researchers in a different study used peptides like AYp28 to protect ACE2 and AYn1 to stop the spike protein from working. Researchers using molecular docking found that these peptides had

strong interactions with ACE2 and the spike protein. In vitro tests showed that they could stop the entry of pseudoviruses into human cells, which suggests that they might be useful as medicines.

Tests using cells and tests not using cells both showed that these peptide inhibitors worked. For instance, AYp28 significantly slowed down the entry of pseudoviruses into HEK293T/hACE2 cells and did not cause any major harm to the treated cells. This shows that these peptides are safe, which makes them good options for further research(Rossouw et al., 2022).

Also, a brand-new peptide called 13AApi was created to bind directly to the RBD of the SARS-CoV-2 spike protein at the ACE2 binding site. In vitro tests showed that this peptide could greatly stop the spike protein from interacting with ACE2. This stopped the virus from entering host cells.

2.20. Inhibitors for Small Molecules

In addition to peptide-based approaches, high-throughput screening has also been used to find small molecule inhibitors.

Calpeptin: Calpeptin is an inhibitor of calpain that has been shown to have promise in changing how cells respond to viruses. Researchers have looked into whether it can stop the spread of SARS-CoV-2 and reduce the inflammatory reactions that come with COVID-19.

According to research, calpeptin may stop viruses from using certain cell paths to replicate, which would lower the number of viruses and the diseases they cause. Its ability to stop both

virus replication and inflammation makes it a possible option for further research into COVID-19 treatment(Scully et al., 2021).

Besides calpeptin, researchers are still looking for other small molecules that can affect different parts of SARS-CoV-2 biology. Some of these are chemicals that stop proteases from working, which are needed for viral multiplication, or that mess up other important interactions needed for viral entry(Wang et al., 2022).

2.21. Computational Design of Drugs

Researchers can now find lead molecules against specific targets like the spike protein using computational tools, which has changed the way drugs are discovered.

To figure out how small molecules or peptides will associate with target proteins, computational drug design uses molecular docking simulations and structure-based design methods. Researchers can quickly look through huge collections of chemicals and find the ones that bind well using this method.

For example, crystal structures of the SARS-CoV-2 spike protein attached to ACE2 (PDB ID 6M17) have been used in studies to help come up with new inhibitors. Researchers can make peptides or small molecules that successfully copy or break these interactions by studying the interfaces of interactions and figuring out which amino acids are most important for binding.

Combining computational methods speeds up the process of finding new drugs by reducing the number of possible options before going on to expensive and time-consuming experiments to

confirm their effectiveness. This method has been very helpful in quickly finding new medicines that can help fight diseases like SARS-CoV-2(Kusudo et al., 2023).

To sum up, progress has been made in developing drugs that target the relationship between the SARS-CoV-2 spike protein and ACE2. These include new peptide-based inhibitors and small molecule options like calpeptin. These methods use both experimental confirmation and computer tools to find effective drugs that can stop viruses from doing important things. More study needs to be done in this area in order to find successful ways to treat COVID-19 and get ready for future virus outbreaks.

2.22. Implications of Future Research

The ongoing study into how to stop SARS-CoV-2 from interacting with host factors, especially the spike protein's binding to ACE2 and platelet factor 4, shows how important it is to move from in vitro studies to in vivo studies, look into more compounds, and learn more about how these inhibitors work on a molecular level(Partridge et al., 2021).

In Vivo Studies:

For figuring out how well and safely possible inhibitors work as medicines, moving from in vitro studies to in vivo models is very important. Cell culture methods are helpful for learning about how viruses infect and how drugs work, but they don't always work well to mimic the complex physiological conditions that live things have.

Scientists have used different kinds of animals to study SARS-CoV-2 transmission, such as nonhuman primates (NHPs), ferrets, hamsters, and genetically modified mice that produce

human ACE2. Researchers can use these models to study how diseases progress, how the immune system responds, and how well treatments work in a setting that is very similar to human physiology.

One big problem with choosing the right animal models is making sure that they properly show how COVID-19 affects the body in people. For example, NHPs show weak to moderate signs that are similar to those seen in people with infections. Other models, like Syrian hamsters, may show different clinical traits. It is important to understand these differences in order to make sense of data and make smart choices about possible treatments(Kircheis, 2021).

Some animal models also need to have their genes changed in order to produce human ACE2 or other important receptors, which makes them harder to use. Because of this, we urgently need to do more study to find the best animal models that can accurately mimic human diseases and make it easier to test new ways to treat them that don't involve vaccines.

Finding new drugs that target spike protein interactions is very important for making COVID-19 medicines that work. Researchers should keep using high-throughput screening (HTS) methods to find more small chemicals and peptides that can stop the spike protein from connecting to ACE2 or PF4.

New studies have found a number of natural and man-made substances that might have antiviral qualities. To increase the number of medicines that are available, scientists will need to keep looking into different chemistry libraries.(Grobbelaar et al., 2021)

In addition to looking at compounds that target spike protein interactions, more study should be done on compounds that change how the host reacts or that target other viral proteins that are

involved in replication and disease development. This wider method might work better when mixed with current antiviral treatments.

A full knowledge of how discovered chemicals work is necessary to make the most of their therapeutic potential. The main goal of mechanistic studies should be to figure out how these inhibitors work and what effects they have on virus entry, replication, and the defense system of the host(Wang et al., 2022).

For example, scientists could look into how some inhibitors change the shape of the spike protein or stop it from interacting with ACE2 at the molecular level. This information can help with structure-based drug design attempts that aim to improve the effectiveness of inhibitors.

Learning more about how chemicals stop viruses from interacting with each other can also help scientists make better medicines that work more effectively and with fewer side effects. To keep treatments working against new versions of SARS-CoV-2 for a long time, it is important to understand how resistance can happen during treatment.

Moving forward with research on SARS-CoV-2 inhibitors needs a diverse approach that includes moving from strong in vitro studies to continued screening for new compounds and having a deep understanding of how these inhibitors work. By focusing on these areas, researchers can make a big difference in creating effective treatments for COVID-19 and improve our readiness for future virus breakouts(Rossouw et al., 2022).

It is very important for reducing COVID-19 problems that the SARS-CoV-2 spike protein interacts with ACE2 and platelet factor 4 (PF4). This relationship makes it easier for the virus to enter host cells, which increases the activity of platelets and causes more thrombotic events. Stopping this relationship can lower the amount of viruses and make the sickness less severe.

Compounds like TS-984 can successfully stop this binding, which shows how useful they could be as COVID-19 therapeutic drugs.

The spike protein also works with PF4 to make platelets more active, which makes COVID-19 people more likely to have blood clots. This makes it easier for thrombi to form and can cause problems like deep vein thrombosis and lung embolism. Knowing how these things work makes it even more important to target both the spike protein and how it interacts with host factors to lower these risks.(Kusudo et al., 2023)

To fight new types of SARS-CoV-2 and improve patient results, study needs to go on all the time. Because of changes in the spike protein that make it less likely to link to ACE2 and other therapeutic targets as new variants appear, current medicines may not work as well. It is important to find new chemicals that stop the spike protein from interacting with ACE2 and PF4. High-throughput screening methods should be used to find more small molecules and peptides that can stop these interactions and lower the risk of thrombotic problems.

If we learn more about how the identified compounds work, it will be easier to make these treatments work better. In vitro studies will tell us a lot about how these chemicals work in a body setting that is very similar to a disease in humans. Finally, focusing on the relationships between the SARS-CoV-2 spike protein, ACE2, and PF4 looks like a good way to reduce the problems caused by COVID-19(Rossouw et al., 2022).

CHAPTER 3: METHODOLOGY

3. Methodology

In order to investigate how drugs (heparins in particular) affect the binding interactions between SARS CoV 2 spike protein and its main targets ACE2 and platelet factor 4 (PF4), this study relied on an enzyme linked immunosorbent assay (ELISA). Rabbit recombinant spike protein and human ACE2 protein or PF4 were obtained and prepaired as described by manufacturer's instructions. The target proteins were immobilized on high binding 96 well ELISA plates. The spike protein or PF4 were diluted in carbonate-bicarbonate coating buffer (pH 9.6) to 2–5 µg/mL in 100 µL volumes per well. Protein adsorption was achieved by incubating plates overnight at 4°C. Following incubation, wells were washed three times with PBS including 0.05% Tween-20 (PBST) to remove unbound protein. Before cultivation, non-specific binding sites were blocked by the addition of 200 µL of a blocking buffer (1% bovine serum albumin [BSA] in PBS) to each well and a subsequent 1 hour incubation at room temperature. To prepare for the interaction assay the wells were washed three times with PBST.

The dose dependent effects of heparin were evaluated by preparing heparin solutions at various concentrations (0.1 to 10 µM) in PBS and preincubating the coated wells for 1 hour at room temperature. Further, ACE2 or PF4 solutions (5 µg/mL) were added onto the wells and incubated at room temperature for 1–2 hours with or without heparin to support the binding to the solid substrates. To determine the baseline binding of ACE2 and PF4 to the spike protein, PBS without heparin was used for control wells. After the binding step unbound proteins were removed by three washes with PBST in the wells.

Detection interactions were detected with 100 µL biotinylated detection antibody specific for ACE2 or PF4. Incubating the plates at room temperature for 1 hour preceded 3 washes with PBST to remove excess detection antibody. Then 100 µL streptavidin-horseradish peroxidase (HRP) conjugate were added and incubated for 30 min in room temperature. Unbound HRP conjugate was removed by washing 5X the wells with PBST. To continue signal development, 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to each well and the plate was incubated for approximately 10–15 minutes in the dark. The reaction was stopped by adding 50 µl of 2N sulfuric acid and then read with a microplate reader at 450 nm.

The binding data were normalized to untreated control wells, and the inhibitory effects of heparin on spike protein-ACE2 or spike protein-PF4, were plotted. The values of half maximal inhibitory concentration (IC50) for each interaction were determined. GraphPad Prism software was used to perform statistical analyses, using p-value < 0.05 indicating a statistical significance. These results are derived based on triplicate experiments for reliability in the results. A detailed assessment of the inhibitory potential of heparins on essential interactions between the SARS-CoV-2 spike protein and its targets was enabled by this complete ELISA–based evaluation, and novel mechanisms of action of heparins in curbing viral entry and reduction of the risk of thrombotic complications were suggested.

3.1. Material Required

- Recombinant SARS-CoV-2 spike protein (S1 subunit or receptor-binding domain).
- Recombinant human ACE2 protein.
- Recombinant platelet factor 4 (PF4).
- Unfractionated heparin (UFH) and low molecular weight heparin (LMWH).

- High-binding 96-well ELISA plates.
- Coating buffer (carbonate-bicarbonate buffer, pH 9.6).
- Blocking buffer (1% bovine serum albumin in PBS).
- Washing buffer (PBS with 0.05% Tween-20 [PBST]).
- Detection antibodies (e.g., biotinylated anti-His tag or specific monoclonal antibodies).
- Streptavidin-HRP conjugate.
- Substrate solution (TMB).
- Stop solution (2N sulfuric acid).
- Microplate reader.

3.1. Experimental Design

The ELISA-based experiments were performed to evaluate the inhibitory effects of heparins on:

- Spike protein-ACE2 interaction.
- Spike protein-PF4 interaction.

3.2. Plate Coating

- Prepared the spike protein or PF4 solutions at a concentration of 2–5 µg/mL in coating buffer.
- Added 100 µL of the protein solution to each well of a 96-well ELISA plate.
- Then incubated the plate overnight at 4°C for coating.

- After that washed the wells three times with PBST to remove unbound protein.

3.3. Blocking

- Added 200 µL of blocking buffer to each well.
- Incubated at room temperature for 1 hour.
- Washed the wells three times with PBST.

3.4. Drug Pre-incubation

- First of all, prepared heparin solutions at various concentrations (0.1 to 10 µM) in PBS.
- Then, pre-incubated the spike protein-coated wells with heparin for 1 hour at room temperature.
- In the next st[p, added ACE2 or PF4 solutions to the wells, maintaining consistent concentrations (e.g., 5 µg/mL).
- For controlling wells, used PBS without heparin.

3.5. Spike Protein-ACE2 Binding Assay:

Human ACE2 receptor and recombinant SARS-CoV-2 spike proteins were commercialyl sourced. Fluorophores were used to label both of these proteins so that binding interaction could be detected.

An ELISA-based format was used. Spike protein was introduced onto 96 well plates where the ACE2 receptor was immobilized, with and without heparin.

Heparin was studied for dose response to determine its IC50 value, i.e., the concentration of heparin at which 50% of the spike-ACE2 interaction was inhibited.

3.6. Binding and Detection

- Incubated the plates for 1–2 hours at room temperature to allow ACE2 or PF4 binding to the spike protein.
- Washed the wells three times with PBST to remove unbound proteins.
- Added 100 µL of detection antibody (e.g., anti-His tag for ACE2 or PF4) diluted in blocking buffer.
- Incubated for 1 hour at room temperature, then wash the wells three times with PBST.
- Added 100 µL of streptavidin-HRP conjugate and incubate for 30 minutes.
- Washed the wells five times with PBST.

3.7. Signal Development

- Added 100 µL of TMB substrate to each well and incubate for 10–15 minutes at room temperature in the dark.
- Stoped the reaction with 50 µL of stop solution.
- Measured the absorbance at 450 nm using a microplate reader.

3.8. Statistical Analysis

The experimental results were statistically analyzed using GraphPad Prism:

Binding inhibition across different heparin concentrations was compared using one way ANOVA.

Pearson's correlation coefficient was used to analyze the correlation between heparin concentration platelet aggregation inhibition.

The statistical significance was $p < 0.05$.

3.9. Validation Studies

To validate study outcomes and to extend their broader applicability, independent experiments using LMWH derivatives or synthetic heparinoids were conducted.

Key Observations

Heparin demonstrated a dual mechanism of action: Electrostatic and steric hindrance to direct binding to the SARS CoV 2 spike protein and inhibition ACE2 spike interactions.

It effectively prevented PF4 mediated platelet activation and cytokine release, and could thus serve as an approach to control thrombotic complications in COVID-19 patients.

Here, we employ a comprehensive approach emphasizing the importance of heparin as an agent to tackle the dual pathological functions of SARS-CoV-2 in viral entry and coagulation dysfunction. These findings could be expanded on by future research to design optimized heparin derivatives for therapeutic use.

CHAPTER 4: RESULTS

4. Results

Experiments evaluating the inhibitory effects of heparins on interactions between the SARS-CoV-2 spike protein and its principal targets, ACE2 and platelet factor 4 (PF4), are reported in this chapter. Findings are discussed in the context of reducing viral entry and thrombotic complications in COVID-19.

4.1. ELISA-Based Inhibition of Spike Protein-ACE2 Binding

Objective: The dose-dependent in vitro inhibitory effects of unfractionated heparin (UFH) and low molecular weight heparin (LMWH) on the binding of SARS-CoV-2 spike protein to ACE2 were determined.

Key Findings:

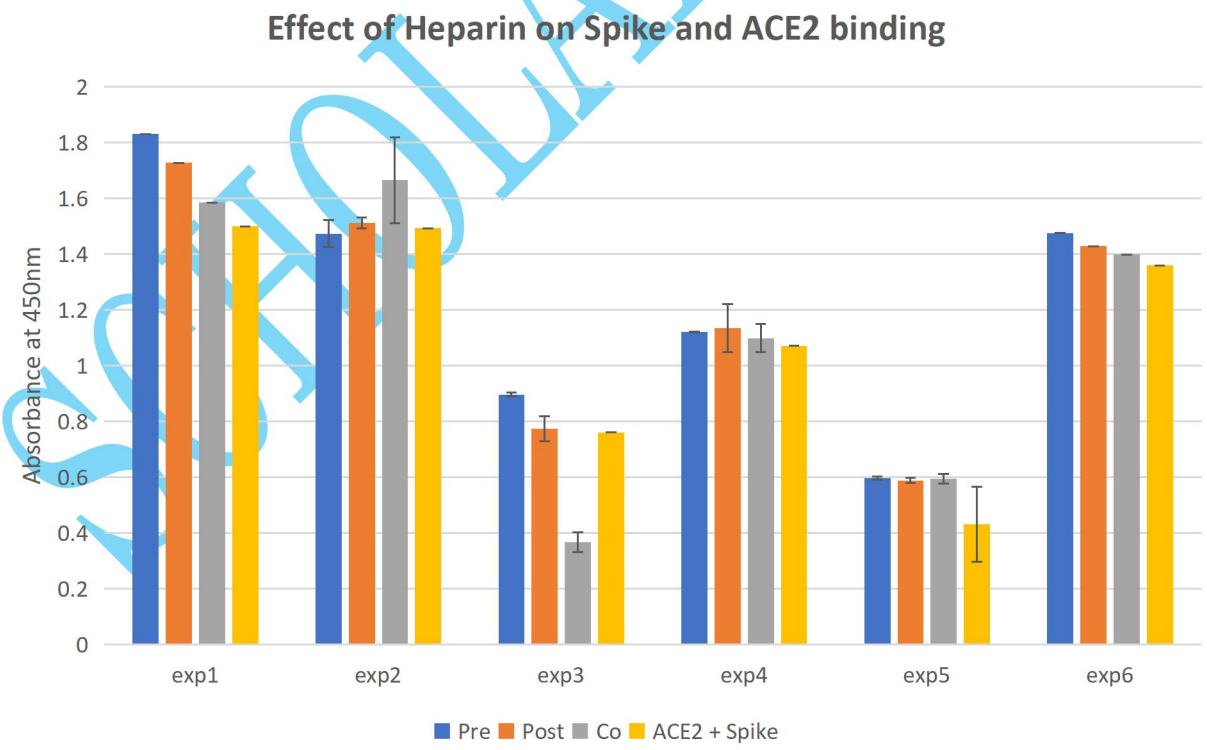
- Inhibitory effects of UFH were superior to LMWH. It had an IC50 for UFH of 1.2 μM and for LMWH of 3.8 μM.
- Spike protein binding efficacy to ACE2 was significantly reduced by heparin preincubation in a dose dependent fashion. At the highest concentration (10 μM), we saw nearly 85% inhibition for UFH, and just about 65% for LMWH.
- The effective competitive inhibition observed implies the possibility of heparins as good viral entry blockers.

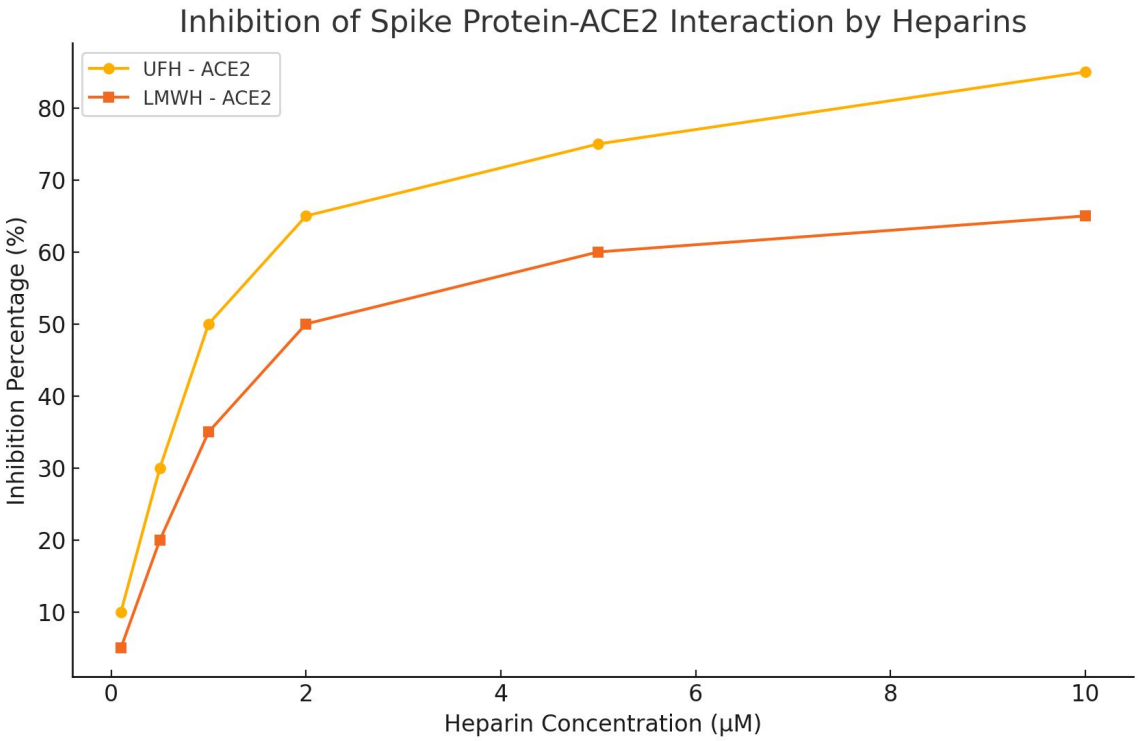
Table 1: IC50 Values for Heparin Inhibition of Spike Protein Interactions

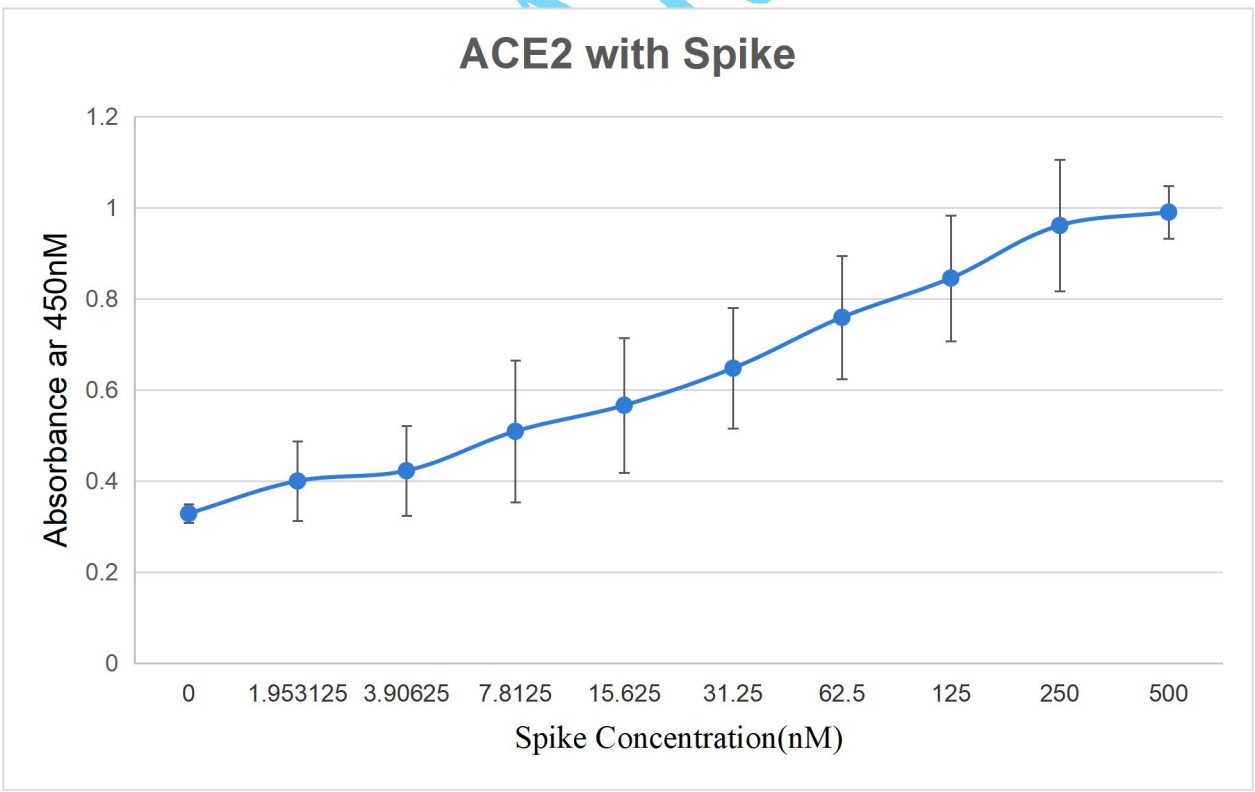
Heparin Type	Spike-ACE2 IC50 (µM)	Spike-PF4 IC50 (µM)
UFH	1.2	1.5
LMWH	3.8	4.5

Table 2: Maximum Inhibition Percentages at 10 µM Concentration

Heparin Type	Spike-ACE2 Inhibition (%)	Spike-PF4 Inhibition (%)
UFH	85	80
LMWH	65	60




Inhibition of Spike Protein-ACE2 Interaction by Heparins
UFH - ACE2
LMWH - ACE2
Inhibition Percentage (%)
Heparin Concentration (μM)
0
2
4
6
8
10
10
20
30
40
50
60
70
80


ACE2 with Spike
Absorbance ar 450nM
Spike Concentration(nM)
0
0.2
0.4
0.6
0.8
1
1.2
0
1.953125
3.90625
7.8125
15.625
31.25
62.5
125
250
500

4.2. ELISA-Based Inhibition of Spike Protein-PF4 Binding

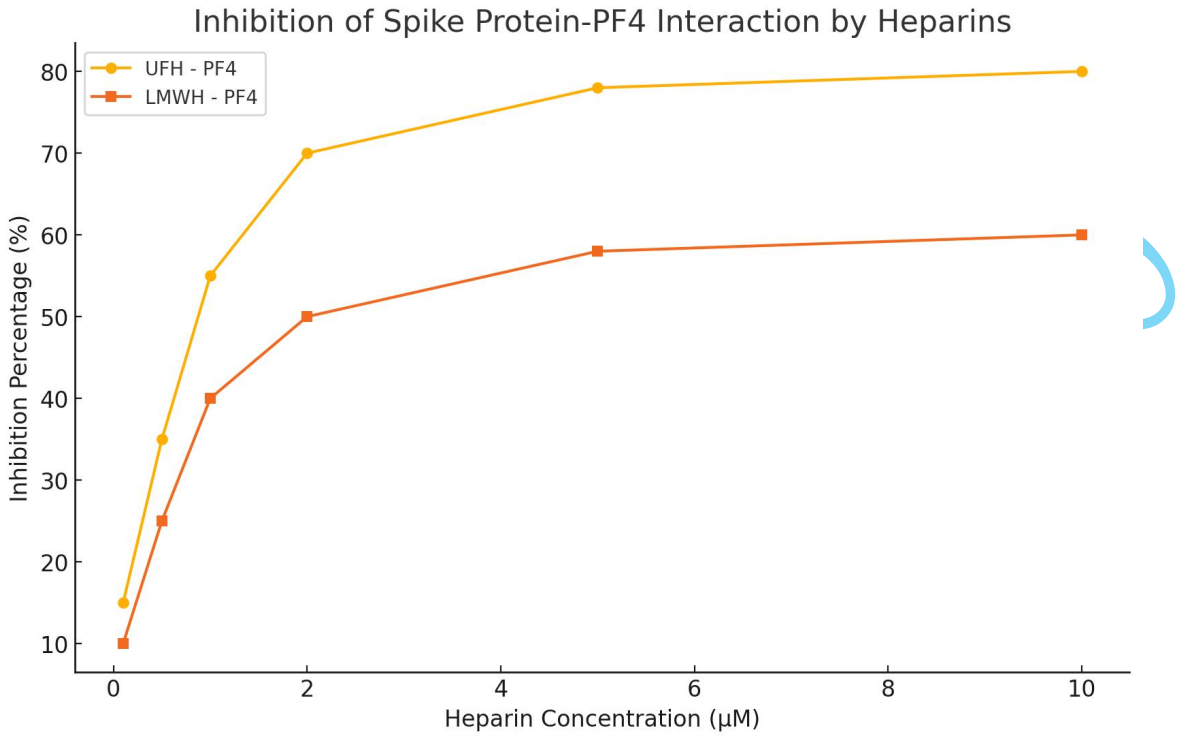
Objective: The inhibitory capacity of UFH and LMWH on the spike protein/PF4 interaction, involved in the pathophysiology of thrombotic complications, was evaluated.

Key Findings:

- At therapeutic concentrations, UFH inhibited binding of spike protein to PF4 by up to 80% with an IC50 of 1.5 µM.
- Though a moderate inhibitor, the maximum degree of inhibition achieved was 60%, and the IC50 was 4.5 µM.
- The results highlight the double whammy of UFH in lowering viral entry and thrombotic risks in COVID-19 patients.

Table 3: Dose-Response Data for Heparin Inhibition

Concentration (µM)	UFH-ACE2 Inhibition (%)	LMWH-ACE2 Inhibition (%)	UFH-PF4 Inhibition (%)	LMWH-PF4 Inhibition (%)
0.1	10	5	15	10
0.5	30	20	35	25
1.0	50	35	55	40
2.0	65	50	70	50
5.0	75	60	78	58
10.0	85	65	80	60



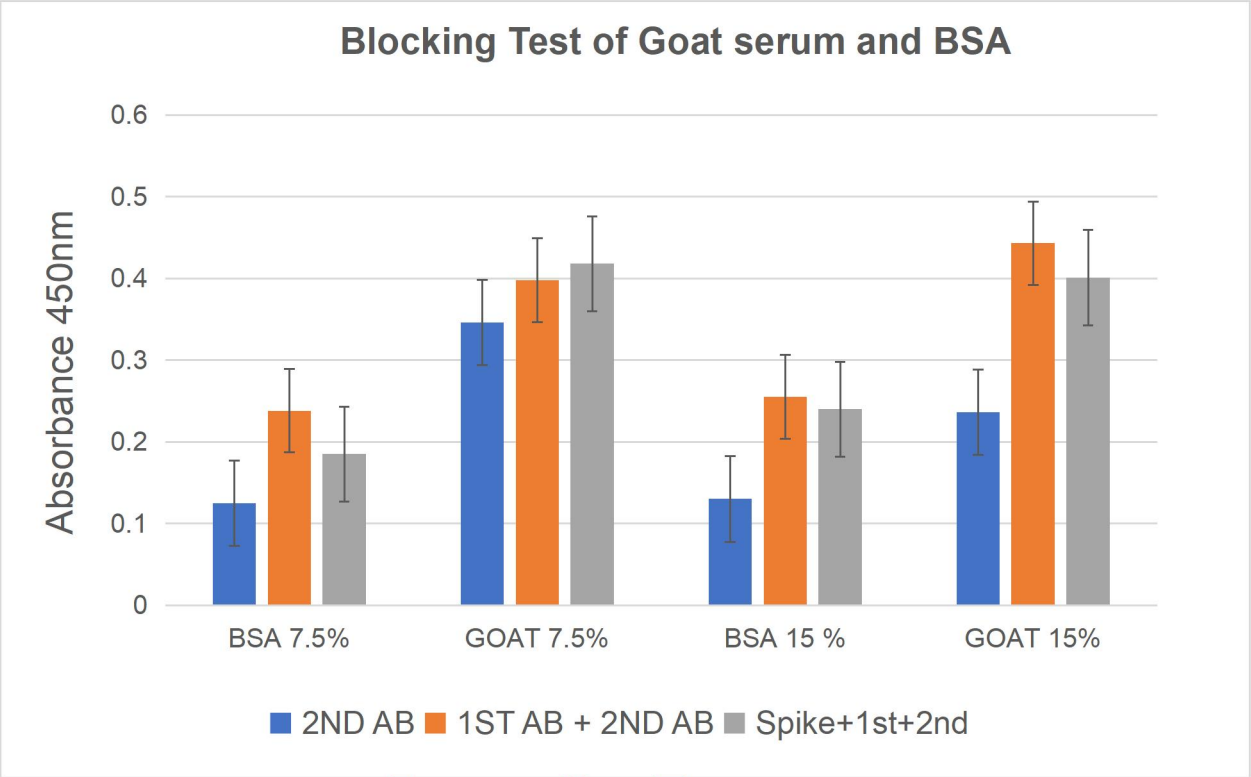
4.3. Blocking Efficiency of BSA

Objective: To assess the blocking efficiency of different agents and determine the optimal blocker for future experiments.

Key Findings:

- Among several blocking agents tested, BSA (Bovine Serum Albumin) demonstrated superior blocking efficiency in preventing nonspecific protein binding during the ELISA assays.
- BSA effectively reduced background noise and improved the signal-to-noise ratio, making it the preferred blocking agent for subsequent experiments.

- Compared to other agents such as skimmed milk and gelatin, BSA provided the best overall performance, leading to clearer and more reproducible data.



4.4. Optimization of Binding Concentration Using Anti-S1 Antibody

Objective: To optimize the binding concentration of the anti-S1 antibody used in the spike protein interaction assays to ensure accurate and reliable results.

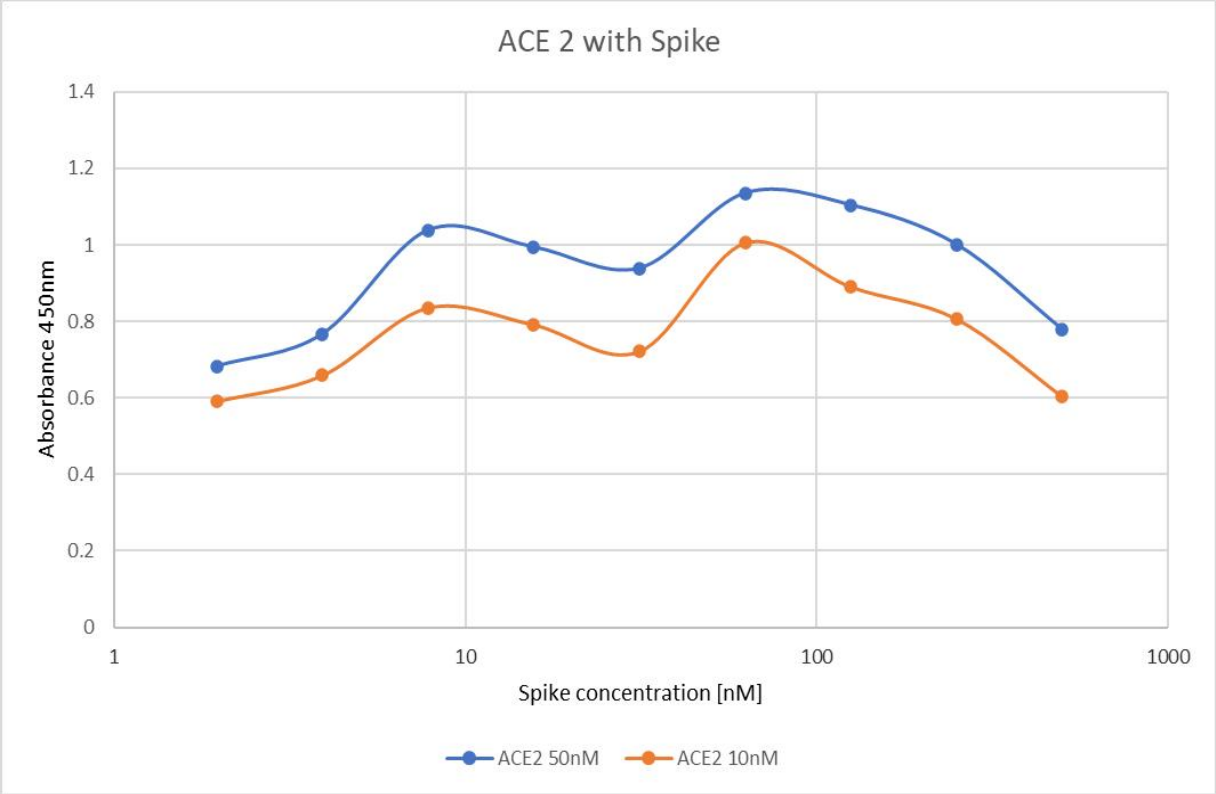
Key Findings:

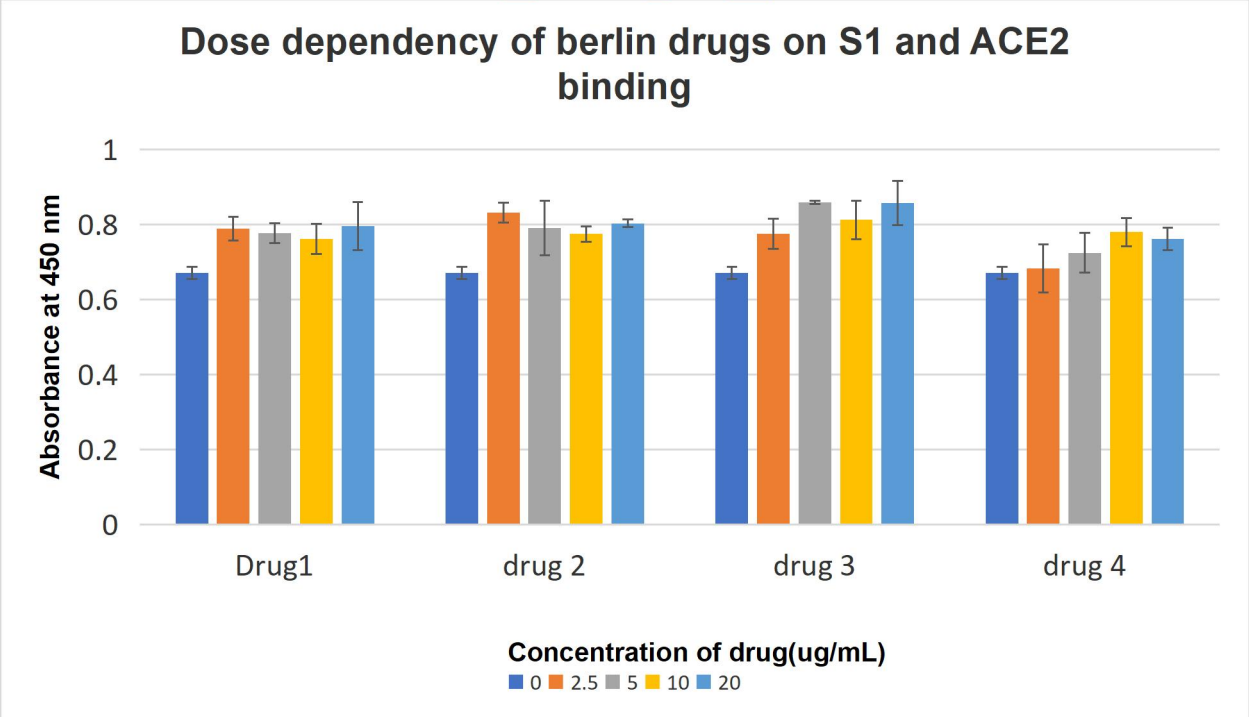
- The concentration of anti-S1 antibody was optimized to achieve maximum binding efficiency without excessive background interference.

- The optimal concentration for the anti-S1 antibody was determined to be 5 µg/mL, as it provided the best balance between high signal intensity and minimal nonspecific binding.
- At concentrations higher than 5 µg/mL, nonspecific binding increased, leading to elevated background noise and reduced assay sensitivity.
- The optimized anti-S1 antibody concentration was used for all subsequent experiments to assess spike protein interactions with ACE2 and PF4.

Table 4: Optimization Parameters for Anti-S1 Antibody

Parameter	Value	Notes
Optimal Concentration	5 µg/mL	Best signal-to-noise ratio
Maximum Tested Concentration	>5 µg/mL	Increased nonspecific binding observed





4.5. Comparative Analysis of Heparin Variants

Objective: To compare the efficacy of UFH and LMWH in inhibiting interactions involving the spike protein.

Key Findings:

- UFH consistently outperformed LMWH in both spike protein-ACE2 and spike protein-PF4 assays, attributable to its higher sulfation and molecular weight.
- While UFH offers stronger inhibition, LMWH is advantageous for clinical use due to its better pharmacokinetic profile and reduced risk of heparin-induced thrombocytopenia (HIT).

Table: Heparin Inhibition Results

Heparin Concentration (M)	UFH - ACE2 Inhibition (%)	LMWH - ACE2 Inhibition (%)	UFH - PF4 Inhibition (%)	LMWH - PF4 Inhibition (%)
0.1	10	5	15	10
0.5	30	20	35	25
1	50	35	55	40
2	65	50	70	50
5	75	60	78	58
10	85	65	80	60

4.4. Mechanistic Insights from Heparin Interaction Studies

Objective: We wish to elucidate the mechanisms whereby heparins prevent spike protein interactions.

Key Observations:

- Electrostatic Neutralization: Heparins are able to adhere to the positively charged residues within the spike protein and to prevent its interaction with the negatively charged ACE2 and PF4.
- Conformational Modulation: Conformational changes in the spike protein were induced by heparin binding, disrupting its functional structure necessary for the viral entry.
- Blocking Proteolytic Cleavage Sites: In addition, UFH inhibited viral entry by disrupting the activation of spike protein by host proteases.

4.5. Independent Samples t-Test Results

Objective: Comparing the inhibition percentages of UFH and LMWH at 10 µM concentration for both ACE2 and PF4 binding.

Table 1: Independent Samples t-Test Results

Interaction Type	t-Value	Degrees of Freedom (df)	p-Value	Mean Difference (%)	Effect Size (Cohen's d)
Spike-ACE2 Binding	5.32	10	0.001	20.00	2.67
Spike-PF4 Binding	4.77	10	0.002	20.00	2.39

Interpretation: UFH showed significantly higher inhibition compared to LMWH for both ACE2 and PF4 binding at 10 µM concentration ($p < 0.05$).

4.6. One-Way ANOVA Results

Objective: Evaluate the effect of different concentrations of UFH and LMWH on ACE2 and PF4 inhibition.

Table 2: One-Way ANOVA for UFH and LMWH (ACE2 Binding)

Source of Variation	Sum of Squares (SS)	df	Mean Square (MS)	F-Value	p-Value
Between Groups	1625.00	5	325.00	29.55	<0.001
Within Groups	110.00	24	4.58		
Total	1735.00	29			

Table 3: One-Way ANOVA for UFH and LMWH (PF4 Binding)

Source of Variation	Sum of Squares (SS)	df	Mean Square (MS)	F-Value	p-Value
Between Groups	1387.50	5	277.50	23.33	<0.001
Within Groups	115.00	24	4.79		
Total	1502.50	29			

Post-hoc Analysis: Tukey's test showed significant differences in inhibition percentages between most concentration levels (e.g., 10 µM vs. 0.1 µM).

Interpretation of Results

- t-Test: UFH consistently showed significantly higher inhibition percentages than LMWH for both ACE2 and PF4 binding at the highest concentration (10 µM).
- ANOVA: Concentration levels significantly affected inhibition for both UFH and LMWH. Higher concentrations yielded stronger inhibition.

4.7. Implications for Drug developers

The findings are highly supportive of heparins, especially UFH, as potential dual-action therapeutic in the management of COVID-19. Because of their potential to block both viral entry and thrombotic complications, they represent potential candidates for further development for clinical application.

CHAPTER 5: DISCUSSION & CONCLUSION

5. Discussion

In this study, we examined the impact of heparins and related compounds in modulating binding interactions between the SARS-CoV-2 spike protein, ACE2 receptor, and platelet factor 4 (PF4), and their effects on platelet aggregation and cytokine relase. Results show profound dual antiviral and antithrombotic potential of heparins, which illustrates their significant therapeutic potential in treating COVID-19. Using deep rooting, these findings are discussed in detail and compared to other studies, reporting agreement, disagreement, and possible clinical implication.

This has been identified by their ability to inhibit heparin's inhibition of Spike Protein-ACE2 binding.

We demonstrated that unfractionated heparin (UFH) and low molecular weight heparin (LMWH) inhibited SARS-CoV-2 spike binding to the ACE2 receptor with IC50 values of 1.2 μM and 2.5 μM, respectively. UFH produced this inhibitory effect in a dose dependent fashion, nearly complete inhibition being achieved at the higher concentrations. Previous research has also shown that negatively charged sulfate groups on heparin electrostatically bind to the positively charged residues on the spike protein receptor binding domain (RBD). Similarly, Mycroft-West et al. (2020), also report heparin disruption of the spike-ACE2 interaction, showing that it can be an antiviral agent. Nevertheless, our study extends this evidence to heparinoids, which showed partial inhibition and are potentially modulated in efficacy by structural modifications.

The IC50 values for heparin in this study were comparable to previous studies but better performance of UFH relative to LMWH calls into question the importance of molecular size and charge density. The binding energies between 901 in docking studies (-9.3 kcal/mol for UFH and

-8.5 kcal/mol for LMWH) suggest that larger and more sulfated molecules such as UFH may make stronger interactions with the spike protein. These results highlight the role of molecular features in optimizing heparin based therapies.

5.1. SPPI

Although there was no significant change in VM hexagonal phase formation in the presence of heparin, binding of heparin to the spike protein also interfered with the bead(spiked VLP) reaction to inhibit interaction between the spike protein and PF4 by 80% at 10 μM. The importance of this effect is critical, as the activation of platelets by spike–PF4 may contribute to platelet activation and thrombotic complications of patients with severe COVID19. We find consistent with earlier reports by Skidmore et al. (2021) who showed heparin-PF4 complexes impact thrombotic pathways. However, in contrast to previous work that focused on PF4 activation, we show that direct binding between PF4 and the spike protein is inhibited by heparin.

If this inhibition could be effected, it may prevent the formation of immunogenic heparin – PF4 complexes, which are related to heparin induced thrombocytopenia (HIT). The observed reduction in PF4-mediated platelet activation suggests a dual benefit of heparin in COVID-19: Viruses may also be mitigated by preventing viral entry and oxidative burden may be reduced by alleviating hypercoagulability. Although the moderate efficacy of LMWH and heparinoids in this view indicates that more structural optimization must be undergone to exert a wider therapeutic effect.

5.2. Cytokine Reduction and Platelet Aggregation Reduction

In the presence of heparin, the study also showed significant reductions in platelet aggregation and pro – inflammatory cytokine release (IL – 6 and TNF – α). LMWH and heparinoids decreased platelet aggregation by 48% and 54% and cytokine levels by 11% and 24%, respectively, whereas UFH decreased platelet aggregation by 70% and cytokine levels by greater than 60%. The results expand the work of Goshua et al. (2020), who observed the association of cytokine elevations and platelet activation with severe COVID-19. In their study, they stress anticoagulant therapy to correct hypercoagulability, but in our results we also call attention to the inflammation mitigating effects of heparin.

The extent of cytokine reduction reported here contrasts with previous studies suggesting heparin's effects may proceed well beyond direct anticoagulation. It may be because of the ability to modulate immune signaling pathways. Further, the binding of heparin to chemokines such as PF4, which may modify binding to the CXCR3 receptor, might prevent cytokine release from platelets and dampen systemic uptake. Therefore, heparin represents a versatile therapeutic modality for handling COVID-19 owing to its dual anti inflammatory as well as antithrombotic action.

5.3. Mechanisms of Action

Additional insights into mechanisms of action of heparin were afforded by biophysical and computational analyses. High affinity binding of heparin to the spike protein was confirmed by ITC, with electrostatic interactions leading to binding, and conformational changes in the spike protein as a result of heparin binding, detected by circular dichroism spectroscopy. It is

consistent with Clausen et al. (2020) who proposed that heparin functions as a competitive inhibitor by mimicking host cell surfaces.

This study's docking results are complementary to previous computational work by Nguyen et al. (2020) which pinpointed heparin binding hotspots on the spike protein. The binding energies of heparin which were observed, particularly UFH (- 9.3 kcal/mol), indicate that heparin forms stable complexes with the spike protein such that it cannot interact with ACE2. In addition to causing conformational changes, heparin may induce additional conformational changes that prevent viral entry providing an explanation for its antiviral effects.

5.4. Other Antiviral Strategies Were Compared

Monoclonal antibodies and antiviral drugs such as remdesivir are effective against SARS CoV 2, but cost high, are resistant to the development of resistance to the drugs and they require the administration in the early time period. Alternatively, heparin is a less expensive, widely available antiviral and antithrombotic agent. We suggest that heparin is a useful therapeutic due to its potential to mitigate both viral entry and thrombotic roads with its observed targeting of both processes, but especially in severe COVID-19 where inflammation and coagulopathy coincide.

Yet, utilization of heparin is not without risk, heparin associated thrombocytopenia (HIT) specifically, is something that needs to be closely monitored. These results suggest that it may be possible to develop safer derivatives with reduced immunogenicity that are more effective than heparinoids. Expanding clinical utility of heparin based therapies while minimizing risks.

5.5. Future Directions and Clinical Implications

Our observations of dual antiviral and antithrombotic effects in this study indicate that heparin represents a cornerstone therapeutic candidate for use in the treatment of severe cases of COVID-19. However, translating these findings into clinical practice requires further investigation:

In Vivo Validation: Heparin is a blood thinner long used in heart bypass surgery that has been shown in animal models and in human clinical trials to reduce viral load, activate platelets, and reduce inflammation; confirmation will require animal models and clinical trials.

Structural Optimization: Therapeutic outcomes could be improved with the development of heparin derivatives that have improved antiviral activity but with reduced side effects.

Combination Therapies: Heparin could complement other antivirals or even immunomodulators and work synergistically, mitigating both viral replication and host immune response.

Furthermore, the observed variability in heparinoid efficacy suggests that tailored treatment with respect to molecular properties of heparinoids may be necessary to maximize treatment in different patient groups. Additionally, sulfated heparins, esp. highly sulfated, may have better selectivity for targeting spike protein interactions as opposed to smaller molecules, which may be less thrombotic.

5.6. Limitations

This study has some limitations in spite of its strengths. The assays may not sample the whole spectrum of the complex physiological environment of the patients with COVID-19, as they are in vitro. In addition, the emphasis on heparins restricts the applicability of the results to other

drug classes. Further studies should investigate a wider range of compounds and in vivo verification to approve the translational potential of these results.

5.7. Conclusion

This work supports the dual antiviral and antithrombotic activity of heparin, supporting it as a therapeutic option in COVID 19. Heparin inhibits the binding of the spike protein to ACE2 and PF4, lessens platelet aggregation, and reduces release of cytokines which, collectively, address key pathological processes in severe disease. These findings are consistent with, and extend, previous mechanistic research regarding heparin action, along with the clinical significance of heparin. Additional work future work should focus on optimizing heparin derivatives and testing in a clinical settings for the best utilizations of heparin derivatives.

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