PRIMER

Understanding Viral Main Protease as a Target for a Potential COVID-19 Treatment; A primer breaking down "Discovery of M Protease Inhibitors Encoded by SARS-CoV-2"

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ABSTRACT

Background: As of the publication of this primer, the SARS-CoV-2 pandemic continues, along with the search for a treatment. Given the urgency of the research, previously tested treatments and compounds are being repurposed with hopes that they will be effective against the virus. Hung et al. (2020) decided to investigate one of these compounds as part of the treatment search. This primer will explore their investigation.

Results: Hung and colleagues investigated the protease inhibitor GC376 as a potential treatment for SARS-CoV-2. GC376 inhibits the viral main protease (Mpro) of SARS-CoV-2, a key protein in viral replication. It was selected as GC376 previously demonstrated effectiveness against another coronavirus, Feline Infectious Peritonitis Virus (FIPV), which has a similar Mpro structure. They observed a stronger binding affinity of GC376 to SARS-CoV-2 Mpro than FIPV Mpro. Additionally, they found an effective dose of GC376 was significantly smaller than the dose needed to induce toxic effects.

Conclusion: Since the publication of Hung et al.'s (2020) article, other researchers have published work on GC376 in the search for a SARS-CoV-2 treatment. Overall, Hung et al.'s (2020) results have been supported, and it has been agreed that GC376 should be further investigated as a potential treatment.

Introduction

In the ongoing SARS-CoV-2 pandemic, finding viable treatments and vaccines are crucial. According to the Center for Disease Control (CDC), as of October 25, 2020, there are 8,553,827 cases of and 224,221 deaths due to SARS-CoV-2 in the United States alone (CDC 2020 Mar 28). SARS-CoV-2 symptoms include shortness of breath, fatigue, body aches, gradual fever, nausea, loss of appetite, loss of taste and smell, and diarrhea (CDC 2020). Additionally, the virus can lead to more serious conditions such as cytokine storm syndrome (CSS) and acute respiratory distress syndrome (ARDS) (Riegelman 2020) (Box 1). Many treatments and vaccines are being tested, but no definite course of treatment or prevention has yet been found. Efforts to create a new drug from scratch to treat SARS-CoV-2 would consume valuable time necessary for treatment. Therefore, many treatments being tested are pre-existing treatments for other conditions, or compounds and proteins known to affect targets present in SARS-CoV-2.

GC376 is one such protein. It is an inhibitor of Viral Main **Protease** (M^{pro}), a protein that is key to viral function. Many viruses initially produce their proteins all together in one long chain of proteins, and the function of M^{pro} is to cleave that long protein into its individual proteins. That way, the proteins become free to perform their intended functions to help the virus survive. FIPV is caused by Feline Coronavirus (FCoV) (Kennedy 2020), a virus in the same family as SARS-CoV-2 (Box 2). FIPV has been under investigation for a long time, as veterinarians still struggle to diagnose and treat it. In clinical trials, GC376

Protease: A protein that cuts other proteins. They are necessary for viruses like SARS-CoV-2, which produce all their proteins all together in one connected string that needs be separated to function.

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cDNA: Complementary DNA is DNA made from an RNA template. A reverse transcriptase enzyme functions to create cDNA.

Cytotoxicity: How toxic a substance is to a host cell.

Mass Spectrometry: The process of measuring the mass-to-charge ratio, which can be used to deduce the chemical signature, mass, and structure of a sample.

Covalent Adduct: When two molecules are attached by a covalent bond.

Transfection: Artificially introducing DNA or RNA into eukaryotic cells.

Vector: A travelling, circular piece of DNA used to pass genes between bacteria.

has previously shown efficacy in treating Feline Infection Peritonitis Virus (FIPV). Cats given GC376 maintained it at an efficacious concentration in their blood plasma to inhibit FIPV M^{pro} (Kim et al. 2016)resulting in substantial change in virulence. Feline enteric coronavirus (FECV. These studies gave Hung et al. (2020) the impetus to investigate GC376 as a potential treatment against SARS-CoV-2 infection.

In their experiment, Hung et al. (2020) wanted to find out how effective GC376 would be at inhibiting the M^{pro} of SARS-CoV-2. They used the viral **cDNA** from SARS-CoV-2 and placed it into *E. coli* to produce the M^{pro} they needed to perform their tests. Afterwards, they did a protease activity assay to observe the binding efficacy of GC376 and other compounds against SARS CoV-2 and FIPV M^{pro}. They also performed assessments of GC376's antiviral abilities and **cytotoxicity** levels to assess whether the inhibitor works to stop M^{pro} from functioning, and whether it would be safe to give a patient a dose of the inhibitor. Next, Hung et al. (2020) used **mass spectrometry** to assess whether a **covalent adduct** formed between GC376 and SARS-CoV-2 M^{pro}. Lastly, they created models of GC376 and M^{pro} from FIPV and SARS-CoV-2 to understand how GC376 binds to each protease. Results indicated that GC376 binds strongly to SARS-CoV-2 protease, is effective in preventing its function, and should be safe to consume at efficacious doses. However, based on mass spectrometry analysis, only 30% of GC376 in solution was bound to SARS-CoV-2 protease, and thus improvements could be made to the inhibitor as a potential treatment.

Methods

Plasmid Transfection into E. coli

In order to test the effect of the GC376 inhibitor on SARS-CoV-2 M^{pro}, Hung et al. (2020) first had to produce SARS-CoV-2 M^{pro} to work with by giving *E. coli* the tools to do so. This was done by preparing M^{pro} cDNA derived from SARS-CoV-2 cDNA. The cDNA was added to a plasmid, chosen for the built in Glutathione-S-Transferase (GST) tag that would allow high purification of the M^{pro} produced. Next, the plasmid was inserted into *E. coli* through **transfection.** Once transfected, the *E. coli* was refreshed and induced to encourage the expression of SARS-CoV-2 M^{pro} from the **vector** (Figure 1).

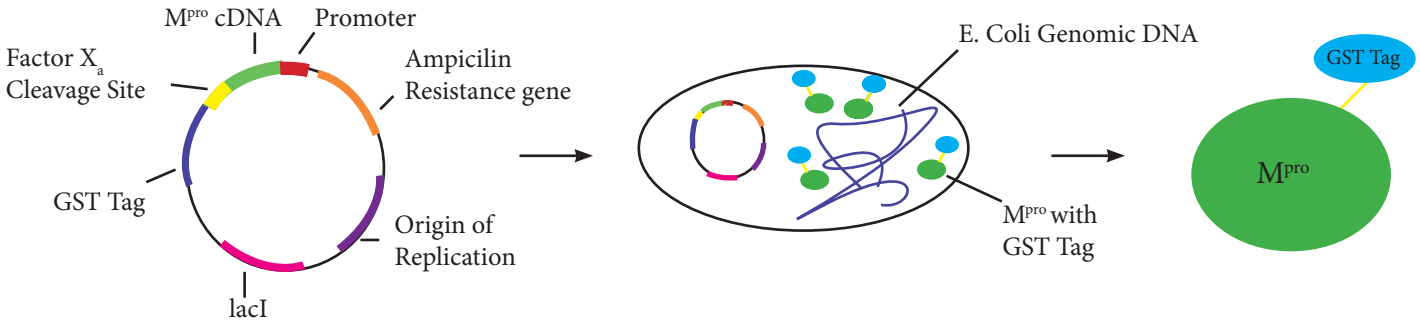

Figure 1: Use of E. coli to produce SARS-CoV-2 Mpro (A) The vector with the Mpro cDNA, promoter, and Factor Xa cleavage site added inside. Includes GST tag for purification, an origin of replication to copy the plasmid, and an ampicilin resistance gene and lacI gene to test for insertion success. (B) Plasmid inserted into E. coli. (C) M^{pro} produced from the plasmid in E. coli.

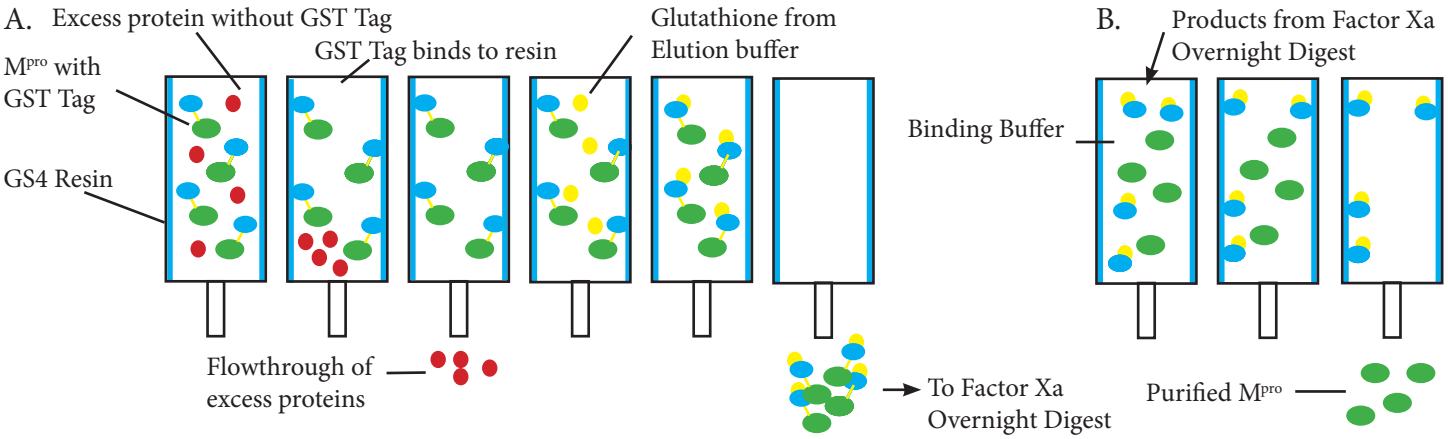

Figure 2: Glutathione Sepharose Column Chromatography to purify SARS-CoV-M^{pro} (A) The protein sample is added to the column with binding buffer, and M^{pro} is allowed to stick to the resin. Unwanted proteins are washed out with the buffer, leaving only M^{pro} in the column. An elution buffer is then used to separate M^{pro} from the resin, and elute it from the column. After an overnight digest with Factor Xa to remove the GST tag from M^{pro} (See Figure 3), (B) the GST tags are removed from solution with the binding buffer, allowing pure M^{pro} to be eluted.

Sonicated: When a sample is agitated through soundwaves.

Chromatography: The separation of a mixture by moving it through a medium where the different components will move at different rates.

SDS-PAGE: A process used for assessing the purity of a protein sample.

FRET Substrate: A fluorescent molecule that will fluoresce upon interaction with select compounds, molecules, or proteins.

IC_{50}: The concentration necessary for a drug to inhibit a target function by 50%.

K_i: The likelihood of a molecule to leave its bond with another molecule.

Purification of M^{pro}

Once grown, the *E. coli* cultures were harvested using centrifugation. This was done to separate the *E. coli* cells from the growth media. Afterwards, the *E. coli* cells were **sonicated** in a lysis buffer, which broke down the cells and begin separating the cells from any SARS-CoV-2 M^{pro} proteins.

After harvesting, the M^{pro} was purified via Glutathione Sepharose Column (GSC) **Chromatography** (Figure 2). This chromatography type was chosen due to its affinity for the GST tag added to M^{pro} in the vector. An overnight digest of factor Xa was used to detach the GST tags from M^{pro} (Figure 3), and the purification buffer was used one more time to remove any loose GST tags from the sample. Hung et al. (2020) then assessed the purity of their M^{pro} samples using **SDS-PAGE**.

Protease Activity Assay

In order to assess the binding capacity and efficacy of GC376 and other compounds against SARS-CoV-2 M^{pro} function, Hung et al. (2020) used a protease activity assay (Figure 4). A preincubation of SARS-CoV-2 M^{pro} was prepared in assay buffer on a microtiter plate with inhibitor and substrate compounds distributed in various concentrations for different wells. Next, a **FRET substrate** (DABCYL-KTSAVLQSGFRKME-EDANS) was added as an indicator for SARS-CoV-2 M^{pro} activity. A fluorometer was then used to assess the microtiter plate, and the data was used in the GraphPad Prism 5.0 program to calculate dose incubation curves, half-maximal inhibitory concentration (**IC_{50}**) values, and dissociation constant (**K_i**) values.

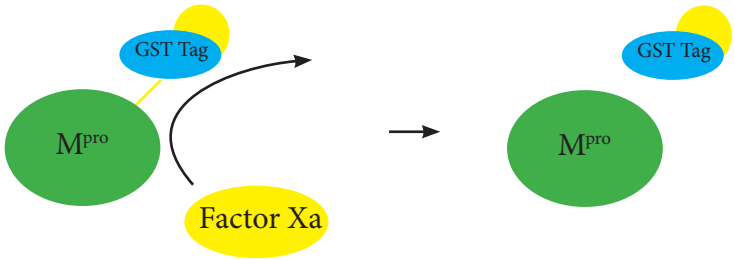

Figure 3: Factor Xa removal of GST tag from M^{pro}

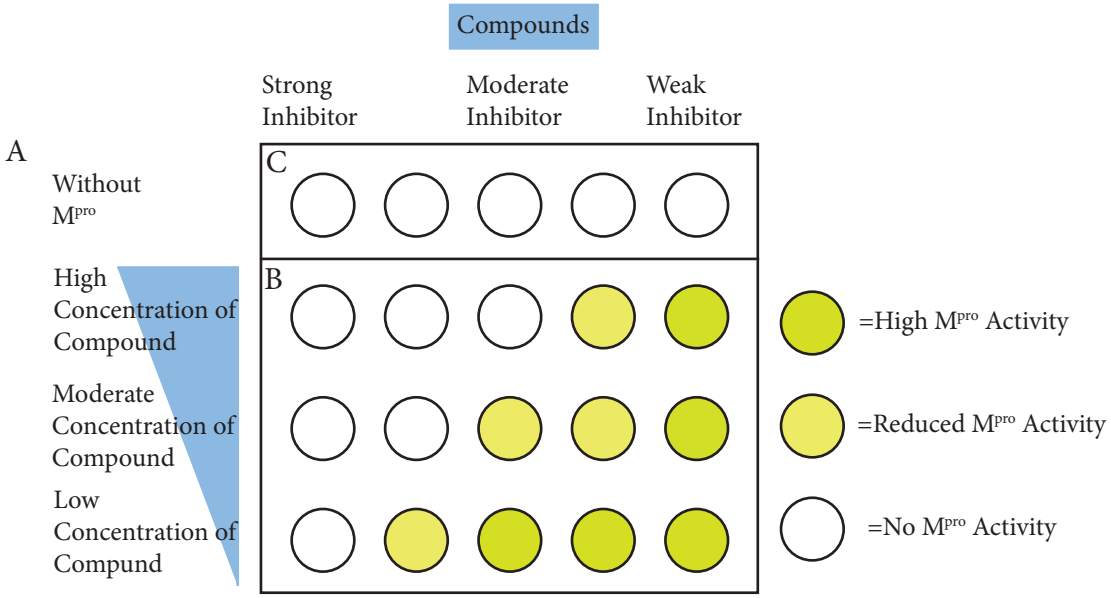

Figure 4: Visualization of the Assessment of Efficacy and Binding Affinity of GC376 and other select compounds against SARS-CoV-2 and FIPV M^{pro} **(A)** Concentration of each compound is decreased with each row. **(B)** M^{pro} activity is indicated by the level of fluoresence shown, as the fluorescent FRET substrate glows in response to being cleaved by M^{pro}. **(C)** Control cell culture without M^{pro}.

Antiviral Assay

To assess the anti-SARS-CoV-2 activity of GC376, Hung et al. (2020) ran an antiviral assay (Figure 5). A 96-well tissue culture plate was then seeded with cell cultures. The cells were incubated, and SARS-CoV-2 and GC376 were then mixed in, with GC376 being added at varied concentrations. After incubation, the cells were fixed and stained. Based on the results of the stain, the half-maximal effective concentration (**EC_{50}**) and IC_{50} levels of GC376 were calculated using the GraphPad Prism 6.0 program. These calculations were done to quantify how effective the GC376 was at inhibiting SARS-CoV-2.

EC_{50}: Concentration of a drug at which the target function will respond halfway between maximal and baseline levels.

MTT Assay: Assay used to measure the metabolic activity of cells, which indicates their viability.

CC_{50}: Concentration of a drug needed to reduce cell viability by 50%.

Cocrystal Structures: Crystal structures are made of at least two components. For example, when GC376 binds to SARS-CoV-2, they form a cocrystal structure.

Cytotoxicity Assay

Hung et al. (2020) used an **MTT assay** to assess the cytotoxicity of GC376. A cell culture was prepared on a microtiter plate similarly to the antiviral assay, but with higher concentrations of GC376 included. MTT dye was used to stain the cells after infection, which indicate the presence of oxidoreductase enzymes, which indicate cell survival. The viability of the treated cells was approximated for each well. From those observations, the 50% cytotoxic concentration (**CC_{50}**) was calculated to quantify the cytotoxicity of GC376.

Molecular Modelling of GC376 Docking to Binding Sites of SARS-CoV-2 and FIPV M^{pro}

To further understand how GC376 docks to binding sites on SARS-CoV-2 and FIPV M^{pro}, Hung et al. (2020) used a modelling program to create molecular models of the proteins and ligand. The binding pocket structure was extrapolated based on Middle East Respiratory Syndrome (MERS) and GC376 **cocrystal structures.** To make the covalent docking calculations, Hung et al. (2020) used the Two Point Attractor method by AutoDock tools 1.5.6.

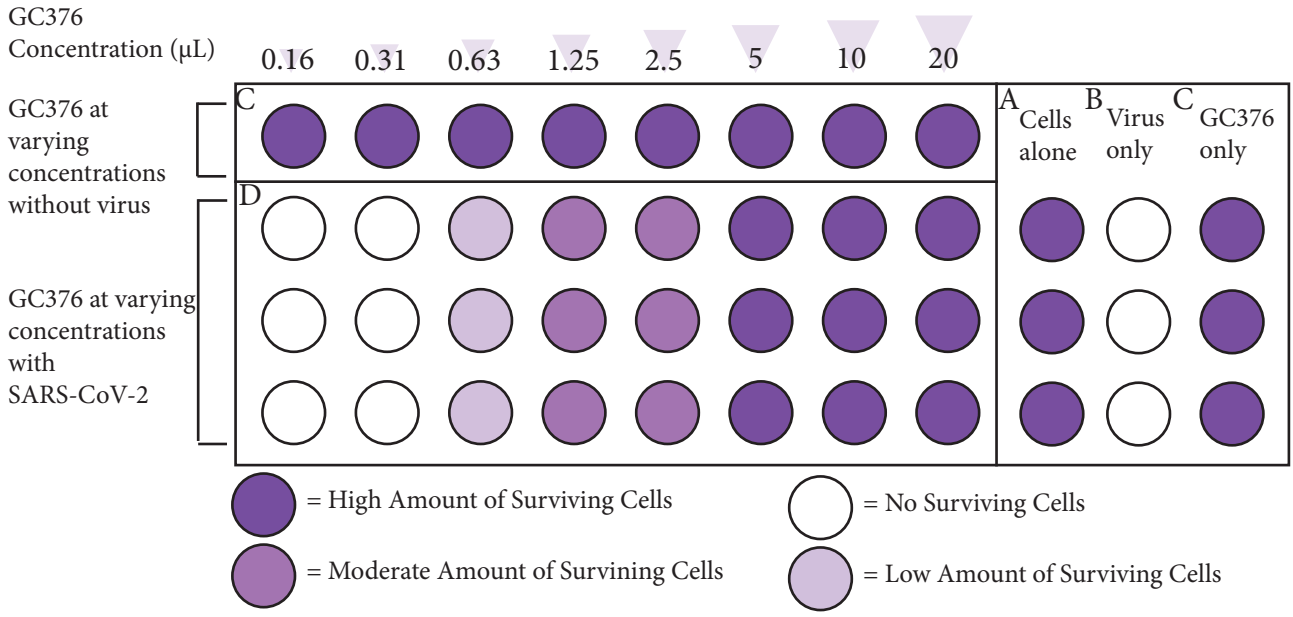

Figure 5: Visualization of the Antiviral Assay to test for the antiviral activity of GC376 against SARS-CoV-2 M^{pro} Crystal violet stains the nuclei of surviving cells, indicating cell survival. Controls are shown for (A) Vero E6 cells alone, (B) with only SARS-CoV-2, (C) and with only GC376. (D) SARS-CoV-2 was then incubated with varying concentrations GC376 to observe its efficacy against SARS-CoV-2.

Mass Spectrometry of GC376-bound SARS-CoV2 M^{pro}

To observe for the possibility of a covalent adduct between GC376 and SARS-CoV-2 M^{pro}, Hung et al. (2020) used a Quadrupole Time of Flight (QTOF) Spectrometer.to assess the weight of the molecules after incubation in solution together.

Results

Hung et al. (2020) hypothesized that GC376 would inhibit SARS-CoV-2 M^{pro} and subsequently inhibit replication of SARS-CoV-2. A protease activity assay was used to determine remaining M^{pro} activity after GC376 treatment (Figure 1). Additionally, an antiviral assay was performed to assess the effectiveness of GC376 against SARS-CoV-2 (Figure 2). A cytotoxicity assay was done on a human cell culture to observe how harmful GC376 would be for humans. Molecular models were created to simulate the binding sites of SARS-CoV-2 and FIPV M^{pro} to assess the difference in their binding to GC376 (Table 1). Lastly, mass spectrometry was used to observe whether GC376 formed a covalent adjunct with SARS-CoV-2 protease, and how frequently the inhibitor binds to the virus.

SARS-CoV-2 Viral Main Protease Activity Assay

As an assessment of anti-SARS-CoV-2 protease activity of GC376 and other choice compounds, Hung et al. (2020) performed a protease activity assay (Figure 4). A 96-well microtiter plate was incubated with SARS-CoV-2 M^{pro} in assay buffer, and different columns were filled with various substrates and inhibitors at decreasing concentrations. A FRET substrate was used as an indicator for SARS-CoV-2 M^{pro} activity to allow observation through a fluorometer of anti-SARS-CoV-2 protease activity from the compounds. The results demonstrated strong binding of GC376 to SARS-CoV-2 protease and effective inhibition of SARS-CoV-2 at a low concentration (Figure 6).

After fluorometer assessment, the numbers were used to calculate the IC_{50} value of GC376 against SARS-CoV-2 M^{pro} to gauge its effectiveness as an inhibitor of the protease.

Table 1: Comparison of Hung et al. (2020)'s Results for SARS-CoV-2 and FIPV Proteases

Value	SARS-CoV-2	FIPV	Conclusion
Binding Affinity (K_i) ± STD	12 ± 1.4 nM	42.5 ± 2.9 nM	GC376 forms a stronger bond with SARS-CoV-2 M^{pro} than FIPV M^{pro}
Free Binding Energy	-51.59 kcal/mol	-32.42 kcal/mol	GC376 has more energy free to bind SARS-CoV-2 M^{pro} than FIPV M^{pro}
IC_{50} ± STD	26.4 ± 1.1 nM	118.9 ± 1.1 nM	Less GC376 is needed to halt 50% of SARS-CoV-2 M^{pro} function
EC_{50} ± STD	0.91 ± 0.03 μM	N/A	About 0.91 μM of GC376 is needed to provide a half-maximal response against SARS-CoV-2 M^{pro}
CC_{50}	>100 μM	N/A	50% cytotoxicity was not seen from GC376 in doses less than 100 μM
Selectivity Index (SI)	>114	N/A	At an effective dose, the antiviral activity of GC376 against SARS-CoV-2 M^{pro} is greater than the cytotoxicity for host cells, and should be safe for consumption

The IC_{50} was calculated to be 26.4 ± 1.1 nM, indicating that to stop half of SARS-CoV-2 protease activity, about 26.4 nM of GC376 is needed. Additionally, binding affinity (Ki) values were calculated to assess how well GC376 bound to SARS-CoV-2 protease. and the calculated K_i value was 12 ± 1.4 nM, indicating a high affinity of GC376 to bind to SARS-CoV-2 protease.

The same assay was done with FIPV M^{pro} as a comparison to the results of the assay with SARS-CoV-2 M^{pro}. The IC_{50} of GC376 against FIPV M^{pro} was calculated to be 118.9 ± 1.1 nM, with a K_i of 42.5 ± 2.9 nM. These values show, respectively, that GC376 forms a stronger bond to SARS-CoV-2 protease than FIPV protease, and a lower concentration of GC376 is needed to stop SARS-CoV-2 protease activity than it does to stop FIPV protease activity.

Other potential inhibitor compounds were tested by Hung et al. (2020) to compare their effectiveness to GC376. The HIV protease inhibitors tested (lopinavir, ritonavir, fosamprenavir, saquinavir, nelfinavir, atazanavir, darunavir, amprenavir, tipranavir, and indinavir), showed no inhibition of SARS-CoV-2 M^{pro}. This was in line with a previous study's findings that lopinavir-ritonavir treatment showed no effect on SARS-CoV-2 M^{pro} (Cao et al. 2020). In comparison, $ZnCl_2$ and $ZnSO_4$ showed complete inhibition of SARS-CoV-2 M^{pro}, supporting previous findings of Zn^{2+} inhibition of SARS-CoV-2 protease (Gawehn et al. 2016).

Antiviral Assay of SARS-CoV-2 M^{pro}

An antiviral assay was done by Hung et al. (2020) to assess the capability to GC376 to halt SARS-CoV-2 M^{pro} activity (Figure 5). Vero E6 cells were cultured in a 96-well microtiter plate and incubated with SARS-CoV-2 M^{pro} and decreasing concentrations of GC376. After fixing and staining, the optical density at 570 nm (**OD_{570}**) values of the well samples were measured to quantify the number of surviving cells left in the culture. Using those values, the EC_{50} of GC376 against SARS-CoV-2 M^{pro} was calculated as a measure of the effectiveness of GC376 against SARS-CoV-2 protease. That EC_{50} value was calculated

OD_{570}: Optical Density, or the amount of light absorbed by a sample in a spectrophotometer when hit with light waves with a 570 nm wavelength.

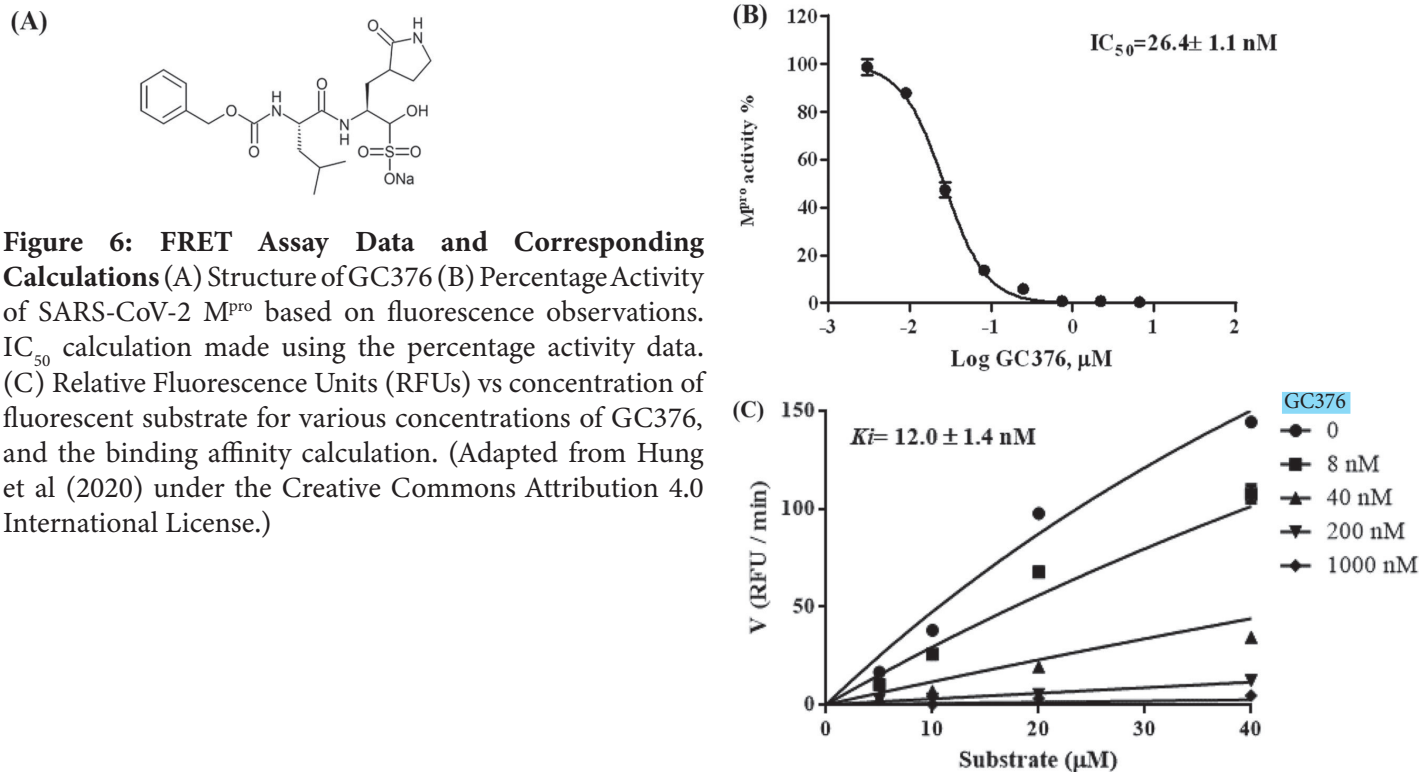

Figure 6: FRET Assay Data and Corresponding Calculations (A) Structure of GC376 (B) Percentage Activity of SARS-CoV-2 M^{pro} based on fluorescence observations. IC_{50} calculation made using the percentage activity data. (C) Relative Fluorescence Units (RFUs) vs concentration of fluorescent substrate for various concentrations of GC376, and the binding affinity calculation. (Adapted from Hung et al (2020) under the Creative Commons Attribution 4.0 International License.)

to be 0.91 ± 0.03 µM, meaning around 0.91 µM is the concentration of GC376 necessary to show a half-maximal response to SARS-CoV2 protease (Figure 7).

Cytotoxicity Assay of SARS-CoV-2 M^{pro}

On a similarly prepared microtiter plate including higher GC376 concentrations, an MTT Assay was done to determine the cytotoxicity of GC376 on a cell culture and investigate a potential safe dose of the inhibitor. GC376 was not observed exhibiting cytotoxicity until it reached a concentration of 100 µM. The **selectivity index** (SI) was determined to be >114, and the calculated CC_{50} value for GC376 was >100 µM. These values mean that GC376 should be safe for consumption and requires a high concentration before producing any cytotoxic effects, respectively.

Selectivity Index: The ratio of antiviral activity to cytotoxic effects. The goal is to have this index be high, as this would indicate that the test compound is more helpful than it is harmful for someone infected with a virus.

Molecular Modelling of SARS-CoV-2 and FIPV M^{pro}

The differences in free binding energies, and in binding of GC376 to different proteases, were compared using molecular models that simulate the docking of GC376 to SARS-CoV-2 and FIPV M^{pro}. Hung et al.'s (2020) observations supported stronger binding of GC376 to SARS-CoV-2 protease than FIPV protease.

To discover which protease GC376 binds tighter, the binding energies of GC376 to SARS-CoV-2 and FIPV M^{pro} were calculated using K_i and IC_{50} values previously calculated. GC376 was found to have a higher free binding energy with SARS-CoV-2 than FIPV with values of -51.59 kcal/mol and -32.42 kcal/mol for SARS-CoV-2 M^{pro} and FIPV M^{pro}, respectively.

For an explanation of the differences in binding affinities and free binding energies, the models were observed for structural differences that affect protease binding to GC376. Only two amino acids were shown to differ between the binding sites. Gln 189 on SARS-CoV-2 M^{pro} is changed to Pro188 on FIPV M^{pro}, which removes a hydrogen bond to the

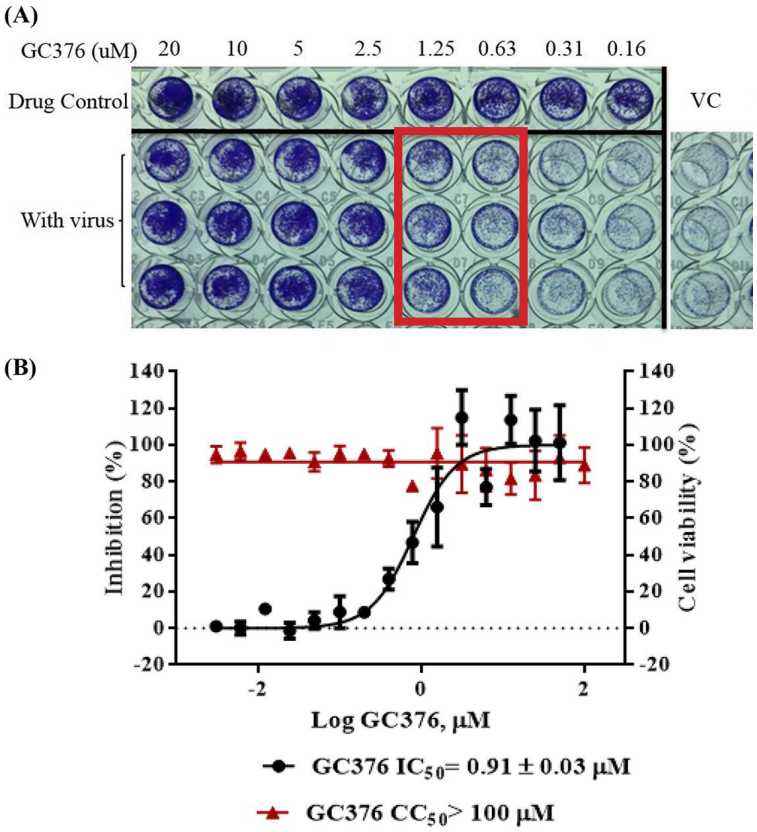

Figure 7: Results of the Antiviral Assay (A) Cell survival begins increasing around 0.63 and 1.25 uM, indicating an estimated minimal effective dose. Controls with cells alone (CC), cells with only virus (VC), and cells with only GC376 (Drug Control) were included for comparison. (B) Cell Viability Data (Red, Right Y-axis) used to estimate the cytotoxicity and EC_{50} of GC376, and M^{pro} Inhibition data (Black, Left Y-axis) including the IC_{50} value (Adapted from Hung et al. (2020) under the Creative Commons Attribution 4.0 International License)

carbamate moiety of GC376, decreasing the bond strength. Additionally, Ser144 in SARS-CoV-2 M^{pro} is swapped with Thr143 in FIPV M^{pro}, but this bond had no effect on the binding network. Moreover, in the GC376/SARS-CoV-2 M^{pro} complex, it was observed that a covalent bond with Cys145 and the hydrogen bond with Gln 189 encouraged stronger binding of GC376 to itself though a strong hydrogen bond network of several amino acids. These observations support the numbers in reflecting stronger binding of GC376 to SARS-CoV-2 M^{pro} than FIPV M^{pro}. Lastly, to explore a possible route of inhibitor improvement, the bisulfite group on GC376 was removed, and that removal led to a new covalent bond with SARS-CoV-2 protease, which would improve the binding strength between the two.

Root mean square deviation (RMSD) values were calculated to determine the similarity of the protease binding sites from SARS-CoV-2 and FIPV. The binding sites were found to be well conserved, with a RMSD value of 1.16 Å, indicating a strong similarity. The SARS-CoV-2 M^{pro}-GC376 complex model was added to NCBI shortly before the publication of the paper, and to assess the accuracy of their binding method, Hung et al. (2020) calculated an RMSD value between their model and the official model. The model was found to have an RMSD of 0.74 Å with Hung et al.'s (2020) model, indicating high accuracy in their modelling method.

Box 1: About SARS-CoV-2

As of the publication of this article, the SARS-CoV-2 pandemic has yet to reach a conclusion, and no treatment or vaccine has been found or completed. SARS-CoV-2 is an acute infection that typically manifests symptoms quickly, albeit not all cases exhibit symptoms. Symptoms of SARS-CoV-2 may include shortness of breath, fatigue, body aches, gradual fever, nausea, loss of appetite, loss of taste and smell, and diarrhea. Depending on the case, symptoms may vary in intensity (Riegelman R. 2020). This includes cases where SARS-CoV-2 causes an extreme inflammatory reaction due to cells releasing too much of specific chemicals called cytokines, which trigger inflammatory responses (Riegelman R. 2020). This is called Cytokine Storm Syndrome (CSS). Additionally, the damage SARS-CoV-2 can do to the lungs can include Acute Respiratry Distress Syndrome (ARDS), a condition where the alveoli responsible for taking up oxygen in the lungs fill with fluid, drastically reducing the oxygen concentration in the patient (Riegelman R. 2020).

Mass Spectrometry of GC376-incubated SARS-CoV-2 M^{pro}

A QTOF spectrometer was used to assess the mass of GC376-incubated SARS-CoV-2 M^{pro} for the formation of a covalent adduct with GC376. Analysis showed a gain of 403.2 Da after SARS-CoV-2 M^{pro} was incubated with GC376, as well as a 34,184.0 Da peak equal to the mass of M^{pro}. This indicates adduct formation and GC376 binding with only one molecule of SARS-CoV-2 M^{pro}. Additionally, peak intensity indicated only 30% of SARS-CoV-2 M^{pro} was conjugated with GC376. This means that 70% of GC376 does not bind to a molecule of SARS-CoV-2 protease, and there is room to improve the binding effectiveness of the inhibitor in a potential treatment.

Discussion

Hung et al.'s (2020) experiment demonstrated the potential of GC376 as a treatment against SARS-Cov-2 through its targeting of viral main protease (M^{pro}). Their data and observations supported the conclusion that GC376 binds more strongly to SARS-CoV-2 protease than FIPV protease. For instance, in their molecular docking analysis, they observed a specific hydrogen bond network in the binding site of SARS-CoV-2 M^{pro}. Additionally, through protease activity, antiviral activity, and cytotoxicity assays, Hung et al. (2020) found support for the safety and efficacy of GC376 as a potential treatment for SARS-CoV-2. However, in their mass spectrometry analysis, they observed that only 30% of GC376 particles became conjugated with SARS-CoV-2 M^{pro}, implying that 70% of the inhibitors present were not binding to SARS-CoV-2 M^{pro}. Using their molecular docking models, they suggested some alterations of the GC376 binding site that could improve its ability to bind and inhibit SARS-CoV-2 M^{pro}.

At the time of publication, the search for a SARS-CoV-2 treatment is ongoing, and GC376 has strong potential to become a treatment for the virus. Not only does GC376 target M^{pro}, a key protein for virus replication, Hung et al. (2020) suspect that GC376 may assist a specific antiviral pathway in the body. This antiviral pathway is the interferon-mediated antiviral system. Interferon, or IFN, is a chemical produced by T-cells and macrophages which, in turn, can activate a cascade of subsequent events which inhibit viral infection. In their previous work, Hung et al. (2011) observed how enterovirus 71 M^{pro} cleaved IRF-9, a key component in the IFN pathway for antiviral activity. The action of an M^{pro} inhibitor like GC376 prevented that IFN pathway interference. This brought them to the idea that GC376 may have a similar effect when treating SARS-CoV-2 (Hung et al. 2020).

Box 2: About FIPV

Feline Infectious Peritonitis Virus (FIPV), as previously mentioned, is caused by Feline Coronavirus (FCoV), a virus in the same family as SARS-CoV-2 (Kennedy 2020). FCoV, depending on the circumstances, may only cause mild to no symptoms instead of causing FIPV infection (Kennedy 2020). This makes FIPV difficult to diagnose, as the causative agent does not always cause the disease, so its presence cannot be used as an indicator for FIPV (Kennedy 2020). A cat infected with FIPV may experience several symptoms, including jaundice, weight loss, loss of appetite, lethargy, fever, central nervous system disease, renal disease, ascites (when the lungs fill with fluid), granulomatous lesions (when immune system cells come together to sequester foreign substances), lymphopenia (viral infection of the lymphatic system), vasculitis (blood vessel inflammation), and enteritis (inflammation of the intestines) (Kennedy 2020; Kim et al. 2016).

Comparison of Effectiveness to the Basis of the Experiment

GC376 has demonstrated potential efficacy against viruses other than SARS-CoV-2. Kim et al (2016) previously tested GC376 as a treatment for FIPV in cats. FIPV is a more virulent version of Feline Coronavirus (FCoV), which typically causes mild to no symptoms. This makes FIPV difficult to detect, as FIPV infection cannot be confirmed by FCoV or antibody presence alone. (Box 2) Of the eight cats Kim et al. (2016) treated with GC376, six survived to a full recovery after 14-20 days without signs of relapse. Additionally, the concentrations of GC376 in the blood plasma were observed rising quickly after administration and maintaining concentrations above the EC50 value for 18 hours afterwards (Kim et al. 2016). The success of GC376 in Kim et al.'s (2016) study, among other information, led Hung et al. (2020) to investigate it as a potential SARS-CoV-2 treatment.

What Hung et al. (2020) observed was GC376 bound stronger to SARS-CoV-2 M^{pro} than FIPV M^{pro}. The Ki values calculated from the protease activity assay for GC376 to SARS-CoV-2 M^{pro} and FIPV M^{pro} supported the stronger binding of GC376 to SARS-CoV-2 (Hung et al. 2020). IC_{50} values calculated from the protease activity assay also indicate a higher effectiveness of GC376 against SARS-CoV-2 protease than FIPV protease (Hung et al. 2020). The molecular docking analysis also showed a stronger binding of GC376 to SARS-CoV-2 M^{pro} through binding site structure observations (Hung et al. 2020). Those observations include a strong hydrogen binding network in SARS-CoV-2 M^{pro} that GC376 was encouraged to bind to through a covalent bond with a specific amino acid absent in FIPV M^{pro}. This would explain the stronger binding of GC376 to SARS-CoV-2, as well as the extra free binding energy Hung et al. (2020) calculated for GC376 with SARS-CoV-2 M^{pro}.

Testing Protease Inhibitors against Other Viruses

GC376 has been previously investigated for action against other coronaviruses. Kim et al. (2016), in addition to their in vivo tests, performed a FRET assay to assess the effectiveness of GC376 against FIPV, SARS-CoV, and MERS-CoV. They found that GC376 was effective against these viruses, being particularly effective against FIPV (Kim et al. 2016)resulting in substantial change in virulence. Feline enteric coronavirus (FECV. This supported the idea that GC376 would be effective against two families of coronaviruses, the alphacoronaviruses, such as FIPV, and the betacoronaviruses, such as SARS-CoV and MERS-CoV (Kim et al. 2016). GC376 has also been investigated for use in pigs to treat Porcine epidemic diarrhea virus (PEDV), a major issue in the swine industry. Ye et al. (2019) found, in their experiment, GC376 was efficacious against two different strains of

PEDV. These are examples of how GC376 has found success against coronaviruses other than SARS-CoV-2.

Other protease inhibitors are being tested as potential SARS-CoV-2 treatments. There have been reports of Ritonavir and Lopinavir, two protease inhibitors usually used against HIV, having antiviral effects against SARS-CoV and MERS-CoV (Ahn et al. 2020). Clinical trials have begun to test these inhibitors against SARS-CoV-2 (Ahn et al. 2020). Additionally, Doi et al. (2020) tested Nafamostat mesylate, previously shown to inhibit MERS-CoV, against SARS-CoV-2. Based on their observations, they determined that Nafamostat mesylate has potential as a treatment against SARS-CoV-2. Lastly, the protease inhibitor metocurine was tested by Jain and Mujwar (2020) against SARS-CoV-2. Ultimately, they determined based on their results that it was a promising lead for a SARS-CoV-2 treatment (Jain and Mujwar 2020). Thus, protease inhibitors have demonstrated potential to yield a SARS-CoV-2 treatment.

CD4 cell count: The number of white blood cells in the blood. A lowered count is indicative of disease.

Protease inhibitors have also been observed as potential treatments for other viruses. For example, Tipranavir was tested by Croom and Keam (2005), in combination with ritonavir, as a treatment for HIV. What they found was an increase in **CD4 cell counts** compared to other drugs paired with Ritonavir (Croom and Keam 2005). Additionally, Lamarre et al (2005) had investigated BILN 2061 as an inhibitor of NS3 protease in Hepatitis C Virus (HCV). After BILN 2061 administration, a reduction in HCV RNA levels in the blood was observed, and Lamarre et al. (2005) found potential in the inhibitor as an HCV treatment. Lastly, Martinez et al (2018) searched for a protease inhibitor for West Nile virus. The protease inhibitor they found was Zafirlukast, and it was found to inhibit West Nile virus NS2B-NS3 protease. Therefore, the idea to research and use protease inhibitors for treatment of medical conditions has an established history in research.

Further Research on GC376 as a Potential SARS-CoV-2 Treatment

RNA Polymerase: The molecule that produces RNA. RNA communicates DNA instructions to the ribosomes, which are responsible for producing proteins.

After the publication of Hung et al.'s (2020) experiment, multiple researchers have begun investigating GC376 alongside them. Fu et al. (2020) performed a series of assays similar in nature to Hung et al's (2020) using GC376 and other potential treatments. Results from their assays supported Hung et al.'s (2020) findings, showing efficacy of GC376 against SARS-CoV-2 (Fu et al. 2020). Additionally, they found that when given together with Remdesivir, an **RNA polymerase** inhibitor, the inhibitory effects of each treatment combined in an additive nature, increasing the effectiveness of treatment (Fu et al. 2020). Combined with previously reported observations of delayed teeth development in cats treated long-term with GC376 (Pedersen et al. 2018)disease signs recurred 1-7 weeks after primary treatment and relapses and new cases were ultimately treated for a minimum of 12 weeks. Relapses no longer responsive to treatment occurred in 13 of these 19 cats within 1-7 weeks of initial or repeat treatment(s, they narrowed down the potential of GC376 to a short-term treatment given with Remdesivir (Fu et al. 2020). SARS-CoV-2 is an acute disease where symptoms arise rapidly, thus any treatment, short-term or not, would be valuable (Fu et al. 2020).

Vuong et al. (2020) also found support for GC376 as a candidate for clinical testing. In their experiment, they calculated an IC_{50} of 0.19 ± 0.05 μM and a CC_{50} value of >200 μM for GC376 acting against SARS-CoV-2 (Vuong et al. 2020). They also tested GC376 against SARS-CoV and found lowered, but present, effectiveness against the virus (Vuong et al. 2020). Comparisons were also made using data from other research testing GC376 against different viruses, and the data showed a broad inhibition of the viruses (Vuong et al. 2020). A strong binding preference of GC376 for SARS-CoV-2 was also observed (Vuong

et al. 2020), which reflects the similar observation made by Hung et al. (2020). Lastly, values from Vuong et al (2020) support the presence of a window between effective and cytotoxic concentrations of GC376 against SARS-CoV-2.

However, Hung et al. (2020) and Vuong et al. (2020) were using Vero E6 cells in their experiment, which are specifically made to be more susceptible to viral infection. Gurard-Levin et al (2020) used HeLa cells and MRC-3 cells, which are not especially vulnerable to viruses, to test the efficacy of GC376, as well as other potential treatments. Using Vero E6 cells as a point of comparison, the assay showed a decrease in this window in HeLa and MRC-5 cell samples through an increase in cytotoxicity. The effective concentration was still small, so it should still be safe to consume at an effective dose. It was the maximum dose that could be safely administered that was decreased.

Most interesting, however is the new method Gurard-Levin et al. (2020) used for their assessments. It is called self-assembled monolayer desorption ionization mass spectrometry (SAMDI-MS), and it is a more sensitive assay that detects, at a higher rate, false positives for inhibition, which occur when test compounds affect optical signals being detected (Gurard-Levin et al. 2020). Other advantages are offered by the method, including removal of enzyme interference from fluorescent tags, ability to reliably test compounds at a wider concentration range, and the increase in compatible buffer compounds (Gurard-Levin et al. 2020). When GC376 and other potential SARS-CoV-2 treatments were tested with SAMDI-MS, the results supported the efficacy of protease inhibitors against SARS-CoV-2, with GC376 being the most effective (Gurard-Levin et al. 2020).

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