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De Novo Designed Peptide to Prevent SARS-CoV-2 Interaction with ACE2 Receptor on Host Cells

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Abstract: COVID-19 pandemic, which has made havoc in the World, is caused by single stranded RNA virus SARS-C0V-2. This virus attacks cells by interacting with Angiotensin-Converting Enzyme 2 (ACE2) receptor on the surface of host cells. Using the Resonant Recognition Model (RRM), we have designed six *de novo* peptides which are proposed to prevent this interaction. Peptide preselection has been by standard Inhibitor Screening Assay Kits and one of the designed peptides (CovA) has been proposed to be a good candidate for testing on cell lines. Using Vero E6 cell line, peptide CovA has shown ability to significantly prevent interaction between SARS-CoV-2 virus and ACE2 receptor. Thus, the designed peptide CovA could provide the basis for development of new COVID-19 drugs. In addition, these results are supporting the RRM model ability to design peptides with desired biological function.

Introduction

Current COVID-19 pandemic has caused havoc in the World, including the number of medical problems from mild symptoms up to severe and critical symptoms, and even deaths, but also the huge economic problems due to worldwide lockdowns. The main problem is that this pandemic was caused by new, up to recently not known virus SARS-CoV-2, which triggered fear how this highly infectious and unknown virus will affect human population. The SARS-CoV-2 virus belongs to large family of coronaviruses, which are single stranded RNA viruses and are widely spread in nature, mostly infecting animals, but some infecting humans as well, usually with mild or non-existing symptoms. Symptoms vary in other species: in chickens they usually cause an upper respiratory tract disease, while in cows and pigs they usually cause diarrhea [1]. However, there have been so far three instances where coronavirus has infected humans, jumping from animals and causing severe symptoms, namely SARS (Severe Acute Respiratory Syndrome, 2003), MERS (Middle East Respiratory Syndrome, 2012) and COVID-19 (2019-nCoV or SARS-CoV-2, 2019 [2]). While SARS and MERS had infected relatively limited areas of population in China and Middle East respectively, they have been less infectious, but with higher mortality rate, while coronavirus SARS-CoV-2 is more infectious, infecting people all around the World, but with much lower mortality rate [3].

As SARS-CoV-2 is, after more than two years, still causing worldwide pandemic, there is urgent need to investigate all aspects of SARS-CoV-2 virus infection and its activity. Although, as there are already developed vaccines, there is still urgent need to design effective and affordable antiviral drugs to prevent or treat COVID-19. In case of coronaviruses, surface spike glycoprotein is first to attach to host cell membrane initiating the infection and could be the most relevant target for design of such drugs [4]. To be able to design drug against SARS-CoV-2, we should firstly identify main characteristic parameters for spike protein interaction with its receptor, Angiotensin-Converting Enzyme 2 (ACE2), and then use these parameters for drug design [4-5]. For that purpose, we have utilised the Resonant Recognition Model (RRM), which proposes that specificity of protein receptor interaction is based on frequency matching between energies distribution along interacting proteins [6-9]. The RRM model is capable to identify protein receptor interaction characteristics and use these characteristics for *de novo* design of peptides with desired biological interaction/function. This approach has been already successfully applied and experimentally tested in the number of examples [10-15].

Here, using the RRM approach, we have identified characteristic parameters (frequencies and phases) for interaction between SARS-CoV-2 spike proteins and ACE2 receptors on host cells. With these parameters

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identified, we have designed six 18-mer peptides with different combinations of these parameters. According to RRM approach, certain combination of parameters is supposed to produce peptide capable to interfere between SARS-CoV-2 spike proteins and ACE2 receptor. To identify such peptide, the peptide preselection has been done by standard Inhibitor Screening Assay Kits and one of the designed peptides (CovA) has been proposed to be a good candidate for testing on cell lines. We have tested here, using Vero E6 cell line, peptide CovA for ability to prevent interaction between SARS-CoV-2 virus and ACE2 receptor on host cells.

Methods and Materials

Resonant Recognition Model

The Resonant Recognition Model (RRM) is biophysical, theoretical model that can analyse interactions between proteins and their targets, which could be other proteins, DNA, RNA, or small molecules. The RRM has been previously published in detail within the number of publications [6-9,16-18]. The RRM model is based on the findings that certain periodicities (frequencies) within the distribution of energy of delocalised electrons along protein backbone are critical for protein biological function and/or interaction with their targets. The distribution of delocalised electrons energies is calculated by assigning each amino acid specific physical parameter representing the energy of delocalised electrons within each amino acid [6-9]. Consequently, the spectral characteristics of such energy distribution (signal) are calculated using the Fourier Transform. It has been shown that proteins with similar biological function and interaction have common frequency component in such spectrum and that this frequency component is characterising their biological function and interaction [6-9]. In addition, it has been also shown that interacting macromolecules have opposite phases at their characteristic RRM recognition frequency [6-9,16-18]. The phase is presented in radians and can be between $-\pi$ and $+\pi$ (-3.14rad and +3.14rad), where the phase difference of or about π (3.14rad) is considered to be opposite.

Once when characteristic frequency and phase for certain interaction between protein and receptor has been identified, it is possible to design *de novo* peptides with this frequency and specific phase, which are possibly capable to interfere with interaction between protein and receptor. This approach has been already successfully applied and experimentally tested in design of FGF analogue [10], HIV envelope protein analogue [11-13] and peptide to mimic myxoma virus oncolytic function [14-15].

Here, we have applied RRM model to find out the coronavirus's common biophysical parameters characterising their binding to the host cell. For that purpose, we have analysed spike proteins (S proteins) from the surface of different coronaviruses and related receptors on the surface of host cells. All sequences were taken from UniProt database as per July 2020: coronavirus spike proteins (QHD43416.1, K9N5Q8, Q5MQD0, Q14EB0, Q0ZME7, P36334, P59594, P11224, P11225, Q8BB25, Q9IKD1, P05135, P11223, Q0Q466, P36300, Q65984, Q7T6T3, P15423, Q6Q1S2, P18450, P33470, P07946, P27655, P24413, Q01977, P10033, Q91AV1) and ACE2 receptors (Q5EGZ1, Q56NL1, Q9BYF1, Q8R0I0, Q59RR0, P21192, Q56H28, Q58DD0, Q5RFN1, Q6FJQ9). Using the RRM approach, we have identified frequency and phase characteristics of SARS-CoV-2 spike protein interaction with ACE2 receptor on the surface of human cells. Consequently, based on these parameters, we have designed six peptides with different combination of characteristic frequencies and phases, namely: CovA, CovB, CovC, CovD, CovE, and CovF as presented in Table 1. These peptides according to RRM principles are possible candidates to be able to prevent analysed interaction and have been *in vitro* experimentally tested here.

Peptide preparation

Peptides CovA, CovB, CovC, CovD, CovE, and CovF (Table 1) were prepared as lyophilized powder by ProteoGenix SAS, France (www.proteogenix.science) and stored at room temperature protected from the light source. All peptides were at least of 95% purity and quality controlled by HPLC and MS by the manufacturer.
Physico-chemical properties, including the estimated solubility of each peptide, were calculated with the peptide property calculator INNOVAGEN (www.PepCalc.com). Peptides with predicted good water solubility (CovB, CovC, CovE, CovF) were dissolved in Phosphate-Buffered Saline (PBS) pH=~7.4. to final peptide concentration of 200µg/mL and were stored at –20°C until use. Two-fold serial dilutions for experimental set up were made in PBS in the range from 0.39 to 200µg/mL.

Peptides with predicted poor water solubility (CovA, CovD) were dissolved in 100%DMSO to final concentration 0.5mg/mL and solutions were stored at –20°C. Before the experiment, the stock solution was diluted 25-fold with MiliQ water to obtain the highest working concentration of the peptide solution 200µg/mL in 4%DMSO and again stored at –20°C. Two-fold serial dilutions were also made in 4%DMSO. Final concentration of DMSO for peptide concentration from 0.39 to 200µg/mL in experimental samples was 0.8%. Peptide solubility was checked with NanoDrop 1000 (ThermoFisher Scientific, US). Similarly, another set of peptides

were prepared in 2%DMSO.
The peptide preparations have been performed at Biotechnical Faculty, University of Ljubljana, Slovenia.

Peptide preselection using Inhibitor Screening Assay Kits (ELISA assay)
The designed peptides were tested for their effects on interaction between the SARS-CoV-2 spike S1 protein and ACE2 receptor using two different Inhibitor Screening Assay Kits (BPS Bioscience San Diego, CA, US), which are based on chemiluminescence production.

Briefly, the Enzyme-Linked Immunosorbent Assay (ELISA) is a microplate-based technique for the detection and quantification of soluble substances such as peptides or proteins. The detection (measured as reaction activity) was based on chemiluminescence due to the substrate reaction with the reporter enzyme. In our assay an inhibitor screening assay kit (BPS Bioscience San Diego, CA, US) was used to screen an effect of potential inhibitors (peptides) on the interaction between the SARS-CoV-2 spike S1 protein and ACE2 receptor.

Interaction with SARS-CoV-2 Spike: ACE2 Inhibitor Screening Assay Kit (BPS Bioscience San Diego, CA, US) was used to test CovD, CovE and CovF peptides according to RRM predictions (Table 1). Prior to the test, peptides were thawed at room temperature for 30 minutes and additionally kept on the thermo block at 37°C for 30 minutes. The peptides were incubated together with ACE2-His and then tested the effect on interaction between ACE2 and spike S1 according to the manufacturer's instructions.

Interaction with ACE2: SARS-CoV-2 Spike Inhibitor Screening Assay Kit (BPS Bioscience San Diego, CA, US) was used to test CovA, CovB and CovC peptides according to RRM predictions (Table 1). The peptides were co-incubated together with the SARS-CoV-2 Spike S1-Biotin and measured the binding of spike S1 to ACE2 according to the manufacturer's instructions.

Blank values for chemiluminescence (e.g., buffers) were subtracted from the experimental values as well as from the controls. To facilitate comparison of different peptides efficacy on the SARS-CoV-2 spike S1 and ACE2 interaction, relative average values and standard deviations were calculated for each peptide to achieve uniformity of the results. The effect of each peptide concentration on the SARS-CoV-2 spike S1 and ACE2 interaction was tested in 8 replicates. Outliers in chemiluminescence intensity were excluded based on the absolute value of modified Z-score > 3.5 as recommended by Iglewicz [19].

Out of six tested peptides tested by Inhibitor Screening Assay Kits, only the CovA peptide inhibited the binding of spike S1 protein to ACE2 receptor in statistically significant manner. The highest effective peptide concentration was 200µg/mL, and the lowest effective peptide concentration was 25µg/mL. The first significant decrease of SARS-CoV-2 spike S1 - ACE2 interaction by CovA was observed at 12.5µ/mL (p=0.031) and 25µg/mL (p <0.0001). The maximum inhibition of SARS-CoV-2 spike S1 – ACE2 interaction by CovA was approximately 80%, which was achieved by 100µg/mL of the peptide. Increasing concentration to 200µg/mL did not further increase the inhibition. Following these results, the testing on cell lines has been done using peptide CovA only.

The peptide preselection experiments have been performed at Biotechnical Faculty, University of Ljubljana, Slovenia.

Cell line and SARS-CoV-2 virus
For the experiments on cell culture, Vero E6 cell line was used. Cells were maintained in Eagle's Minimum Essential Medium (EMEM, ATCC 30-2003) containing 10%FBS (Gibco, ThermoFischer, USA) and 1% antibiotic and antimycotic (Gibco) at 37°C in 5%CO_2 incubator. Cells were routinely tested for mycoplasmas and maintained mycoplasma-free. All work with SARS-CoV-2 and cell culture was performed in a biosafety level 3 laboratory (BSL3) at the Institute of Microbiology and Parasitology (IMP) at Veterinary Faculty, University of Ljubljana. Patient-derived SARS-CoV-2 was provided by European Virus Archive. The stock of virus for further experiments was prepared on Vero E6 cells. The virus titre was determined by titration of the virus on Vero E6 cells and measured as 50% tissue culture infectious dose (TCID50).

Inhibition of live SARS-CoV-2 infection by peptide CovA
Before the experiments with the peptide CovA, the cytotoxic effect of DMSO on cell culture was evaluated by morphological identification of cell death using light microscopy. 4%, 2%, 1%, and 0.5% DMSO in EMEM were tested on Vero E6 cell culture. Vero E6 cells were seeded ($3.5x10^5$ per well) into the 96-well microtiter plate (TPP Techno Plastic Products AG, Switzerland) one day before and incubated at 37°C and 5%CO2. Cells were treated with final DMSO concentrations of 4%, 2%, 1%, and 0.5% in EMEM (8 parallels for each DMSO concentration) and incubated at 37°C and 5%CO2 for 24 hours. DMSO concentrations of 2% or less were found to have no cytotoxic effect on Vero E6 cell culture. Therefore, CovA diluted in EMEM with 2%DMSO was used for further experiments.

The inhibition assay for live SARS-CoV-2 was performed in BSL3 laboratory at IMP. Three different experiments were set: 1) preincubation of peptide CovA with SARS-CoV-2; 2) preincubation of peptide CovA with Vero E6 cells and 3) direct infection of Vero E6 cells with peptide CovA and SARS-CoV-2 suspension. Inhibition activity of peptide CovA was determined by quantitation of intracellular and extracellular virus RNA by the SARS-CoV-2 real-time assay RT-PCR targeting E gene [21]. For all the experiments, Vero E6 cells were seeded ($3.5x10^5$ per well) into the 96-well microtiter plate (TPP Techno Plastic Products AG, Switzerland) one day before and incubated at 37°C under 5%CO_2.

Experiment 1: 100$TCID_{50}$ of SARS-CoV-2 were preincubated with peptide CovA in concentration of 100µM diluted in EMEM with 2%DMSO for 1h at 37°C under 5%CO2. After preincubation, the virus-peptide mixture was transferred to the monolayer of previously washed Vero E6 cells and further incubated for 1h at 37°C under 5%CO_2.
Experiment 2: 100µM peptide CovA diluted in EMEM with 2%DMSO was added to the monolayer of washed Vero E6 cells and preincubated for 1h at 37°C under 5%CO_2. After preincubation, 100$TCID_{50}$ of SARS-CoV-2 in EMEM with 2%DMSO were added and incubated for 1h at 37°C under 5%CO_2.

Experiment 3: prepared mixture containing 100$TCID_{50}$ of SARS-CoV-2 and 100µM of peptide CovA in EMEM with 2%DMSO was straight added to monolayer of washed Vero E6 cells and incubated for 1h at 37°C under 5%CO_2.

In all three experiments, the virus-peptide mixture was removed from monolayer after the incubation, and cells were washed 2 times with EMEM. 100µl of EMEM with 2%DMSO containing 100µM of peptide CovA were added to the cells and incubated for 17h at 37°C under 5%CO_2.

Positive control: 100$TCID_{50}$ of SARS-CoV-2 in EMEM with 2%DMSO were added to monolayer of washed Vero E6 cells and were incubated for 1h at 37°C under 5%CO_2. After the incubation, the virus was removed, and cells were washed 2 times with EMEM. 100µl of EMEM with 2%DMSO were added to the cells and incubated for 17h at 37°C under 5%CO_2.

Negative control: 100µl of EMEM with 2%DMSO was added to the monolayer of washed Vero E6 cells and was incubated for 18h at 37°C under 5%CO_2.
All experiments were done in 6 replicates per experiment.

Quantitation of extracellular and intracellular viral RNA

At 18 hours post-infection, supernatants putatively containing free viruses and infected cells were collected. RNA was isolated from the supernatants and from the infected cells. For the last, the cells were washed 2 times with EMEM after the supernatant was collected. A synthetic trypsin TrypLE™ Express Enzyme (1X), phenol red (Gibco) was used to detach the monolayer. Detached cells in TrypLE™ Express Enzyme served for RNA isolation. RNA was extracted using the MagMAX™ CORE Nucleic Acid Purification Kit on the KingFisher Flex System (Thermo Scientific). Intracellular and extracellular viral RNA was measured by the SARS-CoV-2 real-time assay RT-PCR targeting E gene using primers and probe (E_Sarbeco_F ACAGGTACGTTAATAGTTAATAGCGT, E_Sarbeco_R ATATTGCAGCAGTACGCACACA E_Sarbeco_P1 FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ) [20]. For detection of endogenous control 18S rRNA Applied Biosystems TaqMan Gene Expression Assays, primer limited (Thermo Fisher Scientific, Waltham, USA) contented a pair of unlabelled PCR primers and a TaqMan probe with a dye label (VIC) on the 5' end and a minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end was used. For both RT-PCR assays AgPath-IDTM One-Step RT-PCR Reagents (Thermo Fisher Scientific, Waltham, USA) were used. The PCR volume was 15µl, and it contained 2µl of extracted viral RNA, 7.5µl of 2xRT-PCR, 0.6µl of 25xRT-PCR Enzyme Mix, 400nmol of each PCR primer and 200nmol of probe for detection SARS-CoV2 E gene or 0.83µl 20x Applied Biosystems TaqMan Gene Expression Assays for detection of 18S rRNA, and de-ionized water up to 15µl. The real-time assay RT-PCR was performed with an QuantStudio 3 (Thermo Fisher Scientific, Waltham, USA). Thermal cycling for both assays was performed at 45°C for 10 min for reverse transcription, followed by 95°C for 10 min and then 45 cycles of 95°C for 15s and 58°C for 45s.

Statistics

The statistical significance of ELISA tests was assessed by the Two-Sample t-test (equal variance assumed) using relative luminescence unit (RLU) raw data. Probability values smaller than 0.05 ($p<0.05$) were considered statistically significant. Eight independent replicates were used for all experiments and outliers were excluded from the total average calculations. The data are presented as relative mean values when compared to control values ± the standard deviation of the mean value. The entire analysis was performed using OriginPro 2020 (OriginLab Corporation, Northampton, MA USA). The statistical significances of the results addressing

viral infection were assessed by a one-way ANOVA.

Results and Discussion

RRM Modelling

As the spike proteins, which are on the surface of coronavirus, are the first to approach and recognise host cells, we have analysed spike proteins from different coronaviruses with the aim to find out if there is any common component that can characterise spike proteins. When we analysed whole spike proteins (spike S proteins) from different coronaviruses, as listed in Methods and Materials, using the RRM, the most prominent common RRM frequency has been found at f1=0.2827±0.0009, as presented in Figure 1.

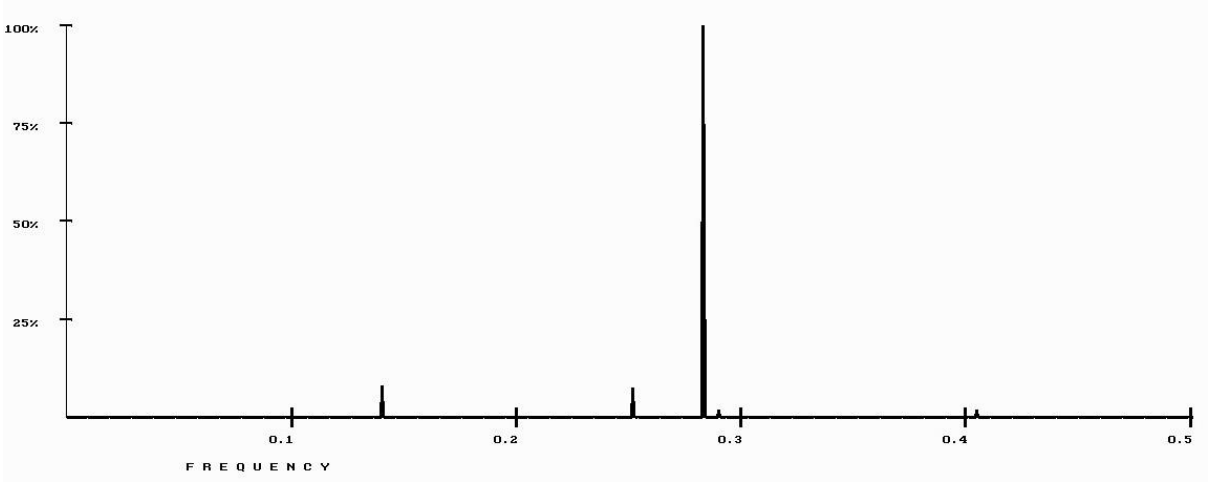

Figure 1. RRM cross-spectrum of spike S proteins with common characteristic frequency is at f1=0.2827±0.0009.

It is interesting to note that there is one unique common characteristic for all analysed spike S proteins from many different coronaviruses, as listed in Methods and Materials. This characteristic could be related to their common biological function.

The first step of viral infection is interaction between spike S1 fragments (spike S1 proteins) and receptors on surface of host cells. The main entry point into host cells for some coronaviruses, including HCoV-NL63, SARS-CoV (the virus that causes SARS) [21] and SARS-CoV-2 (the virus that causes COVID-19) [22] is ACE2, which is attached to outer surface of cell membranes of cells in the lungs, arteries, heart, kidney, and intestines [23]. More specifically, the binding of the spike S1 proteins of SARS-CoV-2 to the enzymatic domain of ACE2 receptor on the surface of cells results in endocytosis and translocation of both the virus and the enzyme into endosomes located within cells [24].

When we have analysed spike S1 proteins from coronaviruses affecting humans, as listed in Methods and Materials, using the RRM, the most prominent common RRM frequency has been found at f2=0.3145±0.0019, as presented in Figure 2.

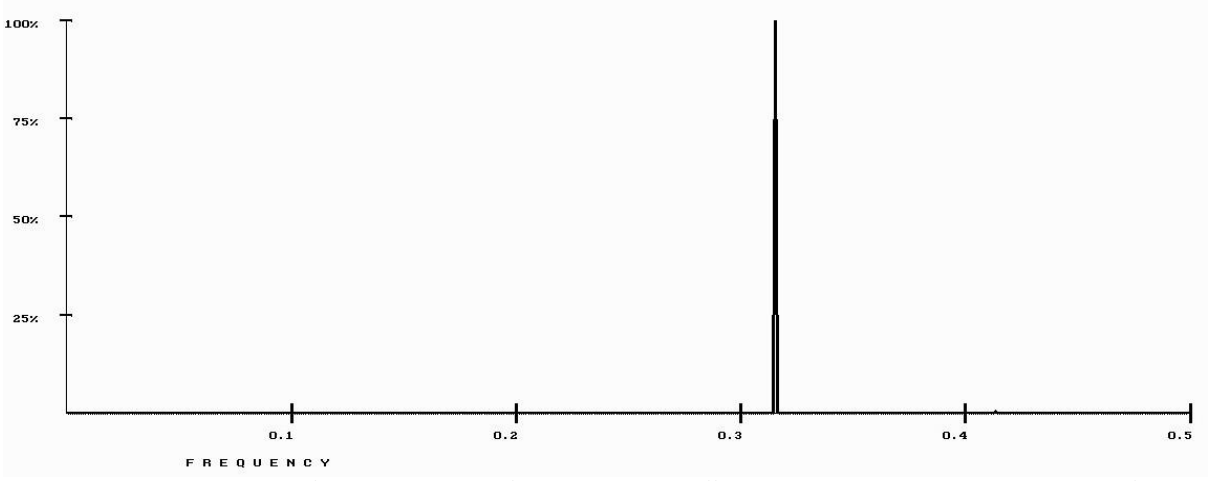

Figure 2. RRM cross-spectrum of spike S1 proteins from coronaviruses affecting humans with common characteristic frequency of f2=0.3145±0.0019.

The next step was to analyse interaction between spike S1 protein and ACE2 receptor to find out RRM frequency characterising this interaction [18]. To achieve this we have compared, using RRM cross-spectra function, ACE2 receptors and spike S1 proteins from coronaviruses that are interacting with

ACE2 receptor, as listed in Methods and Materials. The prominent common characteristic frequency appears to be at the same RRM frequency of f2=0.3145±0.0019 [18].

According to RRM principles, the frequency f2 is characterising the interaction between spike S1 proteins and ACE2 receptors. To confirm that spike S1 protein from SARS-CoV-2 can interact with human ACE2 receptor, we have calculated phases for those two interacting proteins at RRM characteristic frequency of f2=0.3145 to find out if they are opposite, as required by RRM for successful interaction. The phase for spike S1 protein was found to be at -1.74rad, while the phase for human ACE2 receptor was found to be at +2.26rad, indicating that their phase difference is 2.28rad, which is empirically shown to be opposite enough to enable successful interaction [18].

So, we have identified two RRM characteristic frequencies for coronaviruses spike S proteins: f1=0.2827±0.0009 characterising whole spike proteins and f2=0.3145±0.0019 characterising spike S1 proteins and their interaction with ACE2 receptor. As described in RRM methodology it is possible to design peptides having the characteristic frequency(s) and phase(s) related to targeted protein. The phase at frequency f1 for SARS-CoV-2 spike S protein is identified to be at +2.77rad, while the phase at frequency f2 for spike S1 protein of SARS-CoV-2 is identified to be -1.74rad and phase for related human ACE2 receptor at the same frequency f2 is identified to be +2.26rad. Using these results, we have utilised the RRM to design the following six 18-mer SARS-CoV-2 related peptide, as presented in Table 1.

Table 1. List of designed peptides.

Peptide name	**f1=0.2827**	**ph1=+2.77rad**	**f2=0.3145**	**ph2=-1.74rad**
CovA	X	same	X	same
CovB	X	same		
CovC			X	same
CovD	X	opposite	X	opposite
CovE	X	opposite		
CovF			X	opposite

The designed peptides have been tested for preselection using two different Inhibitor Screening Assay Kits and only peptide CovA inhibited the binding of spike S1 protein to ACE2 receptor. Following these preselection results the testing on cell lines has been done using peptide CovA only.

Inhibitory activity of peptide CovA against in vitro infection by live SARS-CoV-2

As the peptide CovA has promising inhibitory activity between SARS-CoV-2 spike S1 protein and ACE2 receptor at around 80%, the further inhibitory activity tests with live SARS-CoV-2 virus on Vero E6 cells were done only with peptide CovA. Therefore, we have tested the ability of CovA to inhibit SARS-CoV-2 infection of Vero E6 cells in three experimental setups:

- experiment 1 with CovA preincubated with SARS-CoV-2 prior to infection.
- experiment 2 with CovA preincubated with Vero E6 cells prior to infection.
- experiment 3 in direct infection Vero E6 cells were treated simultaneously with peptide CovA and SARS-CoV-2 suspension without preincubation.

The results of inhibition assays (Table 2) indicate that CovA inhibited SARS-CoV-2 infection if the peptide was preincubated with Vero E6 cells, as no viral RNA was detected in the cells or supernatants of all 6 replicates 18h post infection (Table 2, experiment 2). Peptide CovA showed no inhibitory effect during direct infection of Vero E6 cells with peptide CovA – SARS-CoV-2 suspension (Table 2, experiment 3) or if peptide CovA was preincubated with SARS-CoV-2 (Table 2, experiment 1). In both experiments 3 and 1, virus RNA was detected in the cells of all 6 replicates 18h post infections. Viral loads determined by cycle threshold (CT) values averaged 28.81 and 26.85 for experiment 3 and 1, respectively (Table 2). Viral RNA was also detected in the cells of all 6 replicates in the positive control 18h post infections (no peptide added) where viral loads determined by CT values averaged 27.14 (Table 2). Statistical analysis by one-way ANOVA showed no significant differences in the viral RNA levels detected in the cells between the groups of experiment 1, experiment 3 and the positive control (p-value>0.05). In experiments 1 and 3, viral RNA was also detected in the supernatants, but with low CT values between 31 and 37. The results of the positive control also showed low viral loads (Table 2). Viral RNA was not detected in the cells or supernatants of the negative control replicates (Table 2). The results of endogenous control of 18S rRNA showed average CT values of 25.61, 26.32, 26.43 for experiments 1 to 3 and 25.93 and 26.47 for positive and negative control, respectively. Statistical analysis by one-way ANOVA showed no significant differences of 18S rRNA values between groups (p-value>0.05).

Table 2. The results of inhibition assay for live SARS-CoV-2 by peptide CovA determined by quantitation of intracellular and extracellular virus RNA and endogenous control 18S rRNA by real-time assay RT-PCR.

	18sRNA in cells	Viral RNA in cells	Viral RNA in supernatants (% of positive replicates) *
Negative control	26.47 ± 0.55	undetermined	0
Positive control	25.93 ± 0.60	27.14 ± 1.54	83.3
Experiment 1	25.61 ± 0.33	26.85 ± 0.51	83.3
Experiment 2	26.32 ± 0.42	undetermined	0
Experiment 3	26.43 ± 0.84	28.81 ± 1.18	50.0

*due to short incubation period of the experiments, the viral load in the supernatants was low (close to limit of detection) and not all replicates showed positive result even in positive control, thus the results are presented as % of of positive replicates.

Based on the results of SARS-CoV and SARS-CoV-2 viral kinetics on Vero E6 cell line, the optimal window for detection of peptide CovA inhibitory activity should be between 12- and 24-hours post infection at the exponential phase of viral replication [25]. As peptide CovA was designed to interfere between viral S protein and ACE2 cell receptors, it should be the most active in the viral infection phase. By proposed mechanism of interference, peptide CovA should lower the number of attached viruses and therefore it should lower the viral RNA load in the cells and consequently in the supernatant of the cell culture, as it has been shown within experiment 2 (Table 2). Even though the peptide CovA has both frequencies f1 and f2 with the same phases as viral spike S1 protein (Table 1) and should therefore interfere with ACE2 cell receptor, two possible mechanism of peptide activity were tested: inhibition of viral – cell interaction by inhibition of the viral protein inhibition and inhibition of viral – cell interaction by inhibition of the cell receptor. When Vero E6 cells were preincubated with the peptide CovA in concentration of 100µM for one hour the viral infection of cells, as measured by intracellular viral RNA, was completely inhibited as viral RNA was not detected in the cells of all 6 replicates 18h post infections (Table 2, experiment 2). However, live SARS-CoV-2 virus pre-treated with the peptide was still efficient to bind and infect the Vero cells. Identical results were also seen when cells were exposed to the virus – peptide suspension sans virus pre-treatment with the CovA peptide (Table 2, experiment 1 and 3). As peptide CovA carries the f1 and f2 frequencies with the same phases as viral spike S1 protein and the results of *in vitro* tests showed inhibitory activity of the peptide when incubated with Vero E6 cells expressing ACE2, it could be suggested that peptide CovA inhibits SARS-CoV-2 adherence to cells by interfering with ACE2, but not with viral spike S1 protein. Although, the presented results are preliminary and further studies, including dose-dependent activity of the peptide and interactions of the peptide and ACE2 should be performed, the peptide CovA could represent a group of SARS-CoV-2 antiviral peptides that inhibit infection by inhibiting the host receptors. Possible advantage of this inhibitory mechanism is that is less prone to viral resistance developed to various antiviral agents

Conclusion

The aim of this research is to design peptide capable to prevent SARS-CoV-2 interaction with ACE2 receptor on host cells. To achieve this, one possible approach is to identify specific biophysical parameters for interaction between SARS-CoV-2 spike proteins and ACE2 receptors on host cells and based on identified parameters to design peptides which could interfere with this interaction. For that purpose, we have utilised Resonant Recognition Model (RRM), which is biophysical model capable to identify specific parameters, critical for protein interaction with its receptor, in terms of frequency matching between energy distribution along the protein [6-9,16-18]. Here, we have identified two RRM characteristic frequencies: at f1=0.2827 characterising coronavirus spike S proteins and at f2=0.3145 characterising interaction between SARS-CoV-2 spike S1 proteins and ACE2 receptors on host cell. In addition, we have identified that phase at frequency f2 for SARS-CoV-2 spike S1 protein is opposite to phase for human ACE2 receptor at same frequency. With frequencies and phases of SARS-CoV-2 spike proteins identified, we have designed six 18-mer peptides with different combinations of frequencies and phases. Using two different Inhibitor Screening Assay Kits only peptide CovA, which has shown strong inhibitory activity of about 80%, has been preselected for cell line testing. As other five designed peptides are not effective, these results are showing that only the very specific combination of RRM frequencies and phases is critical for protein interaction with its receptor. Having identified that the peptide CovA is capable to prevent interaction between SARS-CoV-2 spike S1 protein and ACE2 receptor, we have tested peptide CovA using Vero E6 cells system. With three different experimental settings with Vero E6 cells, it has been shown that peptide CovA interfered with SARS-CoV-2 infection and entry of the virus into the host cell most probably by interfering with the ACE2 receptor.

In this paper, we have shown *in vitro* that designed peptide CovA, can successfully interfere with SARS-CoV-2 infection and prevent entry into the host cell via ACE2 receptor. This peptide CovA has been designed *de novo*, using only RRM modelling

parameters (frequencies and phases), in contrast to currently used homology-based methods for design of peptidic drugs [26]. The results presented here, are not only giving the basis for development of new COVID-19 drugs, but also are supporting the RRM model, as very useful approach for design of novel drugs.

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