Opinion

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Using the Power of Nature Against Pathogens Searching for Antiviral Peptides Effective Against SARS-CoV-2 (In Fish, Bats, and Humans)

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ABSTRACT

Today our society is under increasing threat due to infectious diseases. The current pandemic is just a warning sign of this problem. With the spread of antibiotic resistance, there is a growing need to discover new, effective, antimicrobial drugs. Antimicrobial peptides are "medicines" of nature which may be able to fill the role of lost antibiotics. They can be used not only against bacteria, but also against viruses and fungi. The main purpose of our research is a development of a molecular trap which can separate antimicrobials from hosts which synthetizing them against pathogens. These antimicrobials are produced likely in all living organisms. Our molecular trap is an affinity chromatography method. If it will work, we can develop a new nasal spray vaccine which is optimized on AMPs. We also create new hypothesis how we can design artificial antimicrobial peptides or how we can increase natural peptide's efficiency. We plan to try our methods on SARS-CoV-2, so our results can be immediately applied in the current situation. Our research may therefore have a serious impact of the current pandemic (which is not finished), but also our whole war against pathogens (Findlay, et al. [1]).

Abbreviations: RBD: Receptor Binding Domain; RBM: Receptor Binding Motif; FP: Fluorescence Polarization; BLI: Bio-Layer Interferometry; SPR: Surface Plasmon Resonance; ITC: Isothermal Titration Calorimetry

Introduction

Science knows the existence of these oligopeptides or small proteins since decades, but drug industry interested more seriously in these molecules as a drug just few years ago. (Mahendran, et al. [2]) The number of the peptide drugs increasing. One of them for example is efficient against HIV and was separated from a plant. These peptides will be screened from blood or other fluids of organisms with our molecular trap from any source species (plants or animals). Of course, only if the planned techniques as effective as we hope. Antiviral peptides are natural molecules and as a medicine they are expected to only have a few side effects at least in optimal concentration. (In high concentration they can be cytotoxic.) Therefore, their social acceptance may be wider. In drug industry there are technologies which useful enough to produce peptide drugs in great amount. So, we can provide enough quantity from them for the whole society in the situations of epidemics.

History

A few years ago, I (one of the authors) observed an antimicrobial effect in my ornamental fish farm in the oral cavity of mouth-brooder cichlids. (Figures 1 & 2) My research colleagues helped to develop a pilot study. Three examinations were performed: eggs hatching, bacteriology, and Mass Spectrometry. We can say that based on the results of these experiments; the observation is likely true. This phenomenon is known in biology we found it in the literature, but

little studied. When the pandemic began, we tried to find antivirals in cichlids against SARS-CoV-2. In the mouth cavity we did not find antiviral effects. Likely because eggs have a strong external layer which may save them from viruses. We turned to fish blood for finding drug candidates. Reading the literature, the most important research for us was a mouse experiment. The researchers examine nile tilapia's natural antiviral peptide which were effective against herpesvirus *in vivo* when used as a "drug" in mice. (Hu, et al. [3]). We know then that we have the chance to find drugs against SARS-CoV-2.

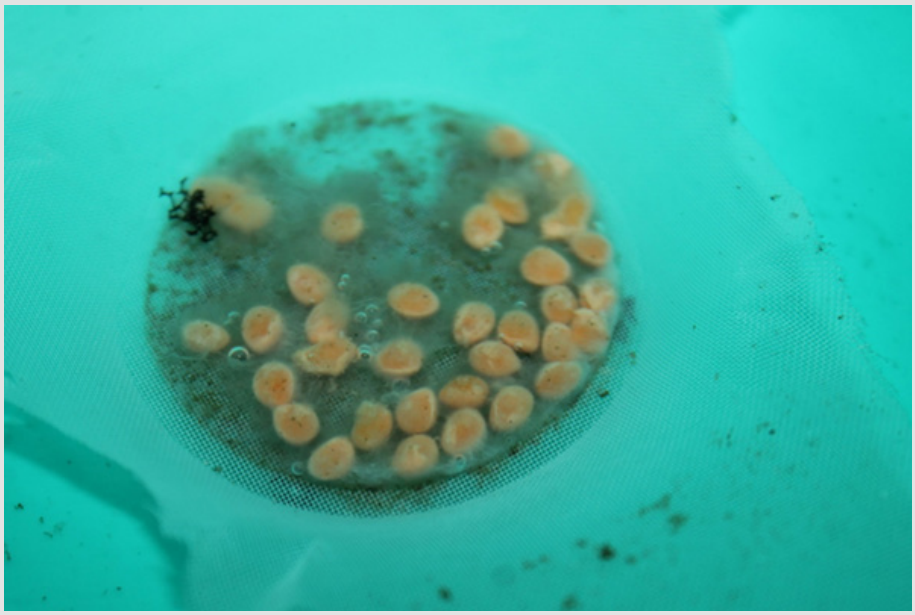

Figure 1: In the photo there are two days old eggs of *Melanochromis johannii* (mouthbrooding cichlid species) in an incubator, eggs were removed from the mouth of the female directly after spawning. All the eggs were died by the attack of microorganisms.

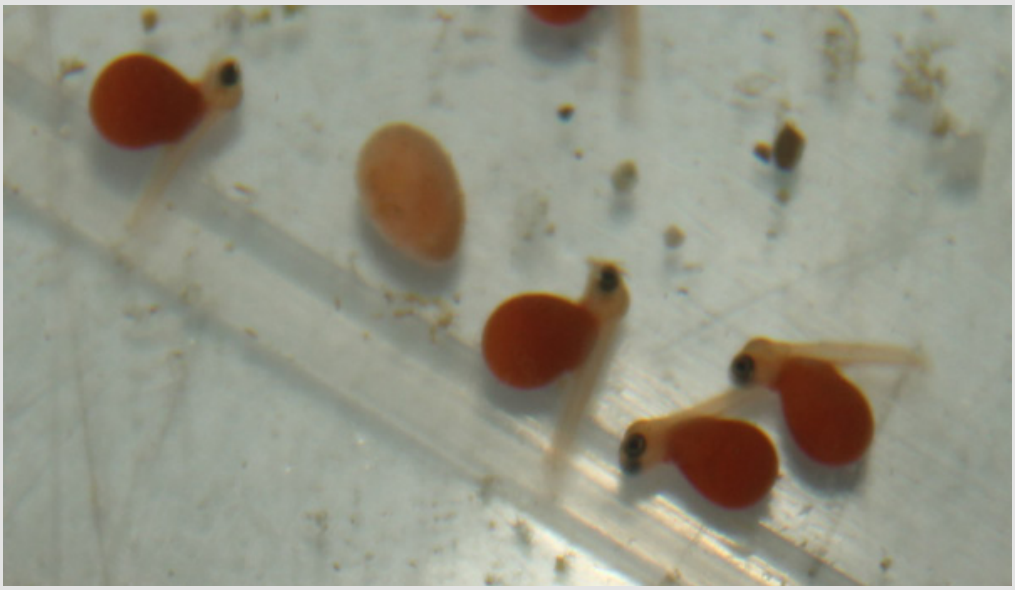

Figure 2: We removed fish larvae from the female *Melanochromis johannii* mouth after 12 days from spawning. We found eggs also, which are not hatched, but remained intact from microorganisms. Based on this we have found obviously a strong antimicrobial effect.

AVP Source Species, in Case of SARS-CoV-2

Lot of antiviral peptides exist. All animal species, but also plant species, produce many of them. (Ramazi, et al. [4]) In the case of SARS-CoV-2, we choose the following source species / group. There were two points of view in the selection: we think we will find the strongest drug candidate molecules in them, and on the other hand we must know the proteome of the selected animals, because it is useful in the structure analysis of the peptides (HPLC-MS).

Goldfish (*Carassius auratus*)

Pathogens spread more easily in the aquatic environment. The immune system of fish is therefore in greater pressure of pathogens. In optimal environment, however, fish's immune system is extremely effective. AVPs are part of the innate immune system which is likely more "effective" in fish than in other Vertebrates. So, we hope we will find particularly effective peptides in their studies. Some of the AVPs are constantly present in the body, others are synthetized just after infections. Therefore, we would like to stimulate the immune system of the fish with inactive viruses in animal experiment, then we can study the peptides which appear as a result

Bats

To the best of our knowledge, the closest relative of SARS-CoV-2 is an Asian horseshoe bat's (*Rhinolophus affinis*) pathogen. (Li, et al. [5]) This virus has similar proteins to SARS-CoV-2, so AVPs produced against them in Asian bats may also be useful in the pandemic. In their natural habitat we can take blood sample from them and examine it with our methods. Coronaviruses are typical "ancient" pathogens of bats, so their antiviral peptides and their production mechanisms may also be highly effective against SARS-CoV-2. Their immune system hypothetically does not kill all the pathogens, just hold their number low and keep balance with them. The survivor viruses maybe continuously activate the immune system of the host. If it is true, it is a marvelous, but honestly dangerous strategy. We would like to examine two bat species: the *Pipistrellus kuhlii* and the Nile flying dogs (*Rousettus aegyptiacus*) in our research. In an animal experiment, we plan to stimulate their AVP production with inactive viruses.

Activating the Production of Human Antiviral Peptides by Nasal Spray Vaccine

More than 100 human antimicrobial peptides have been reported in the literature. (Wang [6]) Based on the statistics of the COVID-19 survivors and thinking over the disease course, it is evident that our body effectively fights against SARS-CoV-2. Therefore, it would be worth searching for antiviral peptides in humans as well. These may be better tolerated by our body. A part of the AVPs just then start to synthetize when pathogens infect us. Our immune system regulates their concentration, so they are efficient but are not cytotoxic. The best we can do to activate their production in our body, and then we do not need to use human AVP-s as a drug. The stimulation of the production of our AVPs (as well as the production of other immune components) using nasal spray vaccines may be the best strategy. (Broadfoot [7]) This can prevent the multiplying of the virus in infected humans by pre-activating the immune system before the pathogen enters our body. Vaccines are designed to produce the right amount of antibody to protect us. Other components of the immune system are activated too. The kinetics of each immune components may be different, many activated immune components may disappear from the body after the four-month vaccination in case of SARS-CoV-2. So, it would be useful to try a different type of vaccination.

Many antiviral peptides production is regulated by molecular signalling systems which are activated by the viruses. In the immune system AVPs primarily function is to prevent the multiplication of the viruses after infection. *In vivo* mouse experiments showed that tilapia's antiviral peptide is effectively protect the animals against herpesvirus. The bioactive peptide provided total protection against this high mortality virus. This effect was experienced for three weeks. (Hu, et al. [3]). It is likely that a dose of a nasal spray vaccine optimized for AVPs will contains significantly smaller number of inactivated viruses like in case of traditional vaccines. But the new vaccine will probably be used more frequently, based on the mouse experiment approximately 2-3 weeks. After vaccination the production of AVPs will begin and this use much less energy than the production of the antibodies in case of traditional vaccination. It means less stress to our body. That's why the side effects would be expected to be even milder than with conventional vaccines. However, it is important that this treatment would not supersede conventional vaccines, but rather complements them. In conclusion, the goal of the new vaccine would be to activate the first line of our immune system for fights against infections.

This may prevent us to be ill and may prevent the spread of the virus in the population. Using the molecular trap we designed, we would be able to detect antiviral molecules that are synthesized in human body by the effect of the nasal spray vaccine. Quantitative changes in AVP's concentration in blood can also be detected with our method. If we know the changes of AVP's concentrations, we can easily determine the quantity of inactive viruses in a dose of the nasal spray vaccine, and the frequency of the vaccine's usage. The SARS-CoV-2 epidemic wave (which shows the number of infected people changes in time) also supports this theory. In case of small number of viruses exist in the population, most people's immune system is not activated, so the virus can spread in the population and begins to multiply in many people. The epidemic wave rises. The immune system of most people will be activated, so further viral infections are inhibited. The epidemic wave starts to decline. The number of the viruses decreases again in humans, the effective immune components in our body will reduce, so the virus can spread in the population and multiply in humans again. The number of infected people rises, and

the next wave begins… With nasal spray vaccines, the formation of wave peaks may be prevented, and perhaps this vaccination method can stop the spreading of future epidemics. The designing and the application of this vaccine can be realized in a short time worldwide.

Molecular Trap

Separation Technique

The searching for AVPs in blood is like looking for a needle in a haystack, it means in this case that blood contains many peptides, but just a few of them have antiviral effects. Finding them therefore is very difficult. We designed a molecular trap; if we would like to find efficient AVPs against a selected pathogen with this method we can screen the bioactive peptides from a source species' blood or other body fluids. We should separate molecules smaller than 30,000 Daltons from the blood sample with micro centrifugation. Our molecular trap is an affinity chromatographic method which is basically used in immunology with antibody-antigen. We bind inactive viruses or their proteins to magnetic beads. If we add the sample's fraction to the trap there will be molecules which attach to the virus proteins, others will not stay in the column. Among the former will be the bioactive molecules that are important to us, the antiviral peptides too. Attached molecules will be released by acid-glycine treatment, but likely still not all of them will be efficient antivirals. We develop the following method to separate further the bioactive ones.

Stimulating the Production of AVPs

Antiviral peptides are not produced continuously. Most of them are expressed in cells only when molecular signaling systems detect the pathogens. To stimulate the production of AVPs we should vaccinate the AVP source organisms with inactive viruses. We plan to take blood samples before and after vaccination. In the latter case we sample for three days in every two hours. So, we can detect changes in AVPs concentrations in blood with the help of the next chemical method.

HPLC-MS Analysis

We identify the components and the concentrations in the samples with HPLC-MS. We will see on the spectrograms which molecules production is started or which molecules concentrations are elevated after vaccination. These components will be worth to research because in this group of molecules will be the efficient AVPs against the virus. With this method we can also determine the structure of these molecules. In AVP's case we must know just the amino acid sequences.

Peptide Synthetization and Testing their Effects on SARS-CoV-2

Depending on the number of AVP candidate's amino acids, we would synthesize peptides and small proteins by biotechnological or chemical methods. Then we will perform virus neutralization tests on the AVP candidates. From the results we will know about the molecules whether they are drug candidates or aren't? (Table 1).

Table 1: Summary table of the molecular trap's research plan.

Animal experiment	Vaccination of the source animals with inactive viruses. Blood samples is taken before and after vaccination, and a fraction of molecules smaller than 30,000 Dalton is separated from the blood sample.
Molecular trap	We bind inactive viruses to magnetic beads and the fraction of the blood sample is added to this affinity chromatographic system. The attached molecules will be washed with acid-glycine treatment.
HPLC-MS	We can detect qualitative and quantitative differences between samples by HPLC-MS. The AA sequences of the interesting peptides will be determined.
Synthesis	These molecules are synthesized by chemical or biotechnological methods.
Virology	We perform virus neutralization tests on these peptides to examine their efficacy.

New Possibilities of Designing Artificial Peptides

Introduction

The efficacy of antiviral peptides is depended on two important questions: how strong the chemical bond between the peptide and the viral protein, and their cytotoxicity. Antiviral peptides can attach to human proteins too (all our proteins mean the proteome) as part of their cytotoxic effect. They can cause changes in enzymes and other proteins and damage them. If their concentration is too high, they become toxic.

New Theory of Artificial Peptide Design

The amino acid sequences of the human proteins (proteome) are known. If we compare the AA sequences of our proteins to the SARS-CoV-2 membrane proteins sequences, we will probably find regions which show significant differences (we need around 20-30 AAs long). If we design an artificial AVP which can bind to the selected viral sequences, then the peptide will theoretically non or just weakly bind to human's proteins, so it's cytotoxic effect will be low. Thus, we can apply peptides as medicine in higher concentrations, which is much better for therapeutic purposes. Beside the sequence, another

important question is where should dock the AVP on the viral protein? The region of the spike protein which binds to the human cell's ACE2 receptor may be most suitable. We call this region of the spike protein as the Receptor Binding Motif (RBM), which is a part of the Receptor Binding Domain (RBD). If a small artificial peptide dock here, there is a good chance that it will prevent the virus from binding to the human receptor-protein and therefore will not be able to enter the cell. (Lan, et al. [8]). We looked for regions within the RBM, we would have liked to find sequences which does not like human proteome. We found that the entire AA sequence of RBD has not so much similarity to human proteome.

This sounds good, but we should keep the next in mind: many antiviral peptides have been designed in silico, working *in vitro* well but to the best of our knowledge, none of them have been used as drugs *in vivo* successfully yet. (Bahr, et al. [9]) Maybe they were strongly cytotoxic. Therefore, we should minimize this, the cytotoxicity of the artificial peptides. For this purpose, we created the "principle of exclusivity" theory. It means we should design our artificial peptides such manner that they can attach exclusively to their docking site on the virus proteins. In optimal case all the artificial AVP's amino acids bind to the spike protein AAs and create binding AA pairs. We should plan the pairs that the peptide's AAs most exclusively attach to the viral AA member of the pair. If there are AAs which bind stronger but less exclusively to the viral AA, we must choose the former, so which more exclusively bind to the virus AA. This resembles to key-lock, our opinion this can be the best way to design the most efficient structure of AVPs with together optimal the two factors: minimize the cytotoxicity of artificial AVPs as much as possible beside the maximization of the strength of protein-peptide bonds. The artificial antiviral peptides can bind to the viral proteins primarily through secondary binding forces between the AA sidechains. Covalent bonds may also play a role, e.g., disulphide bridges. For better results, we can use modified AAs. The criterion in their case that they should be degradable by the human body.

The Structure of Natural Peptides Can Be Changed by The Principle of Exclusivity

This theory can be also combined with the molecular trap method. If the natural AVPs are screened by the trap and modified according to our artificial peptide theory, we may increase their efficacy. We need to find the binding site of the peptide on the virus protein and determine this region's sequence. Then using the exclusivity theory, we can easily redesign the natural peptides. This process also promises the lower cytotoxicity and the maximization of the binding strength of AVPs.

Planned Experiments

To prove our theory, we should first examine the bond strengths between peptides and viral proteins. If we know these, we will better understand the effect of peptides on proteins, so their design will be easier. The preferred test method can be the Fluorescence Polarization (FP), which we can measure the strength of bonds when a small ligand (peptide) binds to a larger one (protein). The peptide must be fluorescently labeled (it can also be done with synthetic and recombinant peptides). It is a fast method that requires a small amount of material. We will have a robust result if we also measure with an orthologous method (the same binding parameter on a different basis), which is either can be the Bio-Layer Interferometry (BLI), Surface Plasmon Resonance (SPR) or Isothermal Titration Calorimetry (ITC).

The Potential of the Methods

These methods can be used in case of most bacterial, fungal, and viral pathogens, revolutionizing our defense against them. We may invent countless antimicrobial molecules. The three methods can be combined. We can use the molecular trap to develop the new vaccine, and to find natural antimicrobial peptides. We can redesign natural AVPs structure with the principle of exclusivity, the artificial AVP theory to make them more efficient and with this theory we can design new artificial AVPs too. AVPs effect independently, so we can apply them together. In this case they can cure more efficient and maybe can treat patients with severe illness. The nasal spray vaccine will be applied beside AVP drugs. This vaccine can prevent the development of illnesses in humans and can stop the epidemics. If we use the three methods together, we may handle the current or future human and animal pathogens much more effectively than in the past. If our theoretical methods will work well, millions of people may be saved yearly. On the other hand, it may be a great negative impact on the drug industry. Politicians and other decision-makers therefore must help this sector because that it is the right, and we need their help. In the end I would like to tell my practical experiences about epidemics and illnesses in my fish breeding system. It is obvious for me after kept hundreds of thousand fish that the best way to prevent populations from epidemics is the good condition of the animals, the ideal keeping circumstances, which do not mean sterility rather natural accommodations. Sounds like evidence but my opinion: this is still the best method we can apply.

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