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Multi-targeted siRNA against conserved genomic regions of flu: new therapeutics with broad activity against emerging flu strains

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PNNL T2-307
CRDF RUB2-40000-ST11 Final Project Report

Institute of Highly Pure Biopreparation & Research Institute of Influenza , Saint Petersburg, Russia

Multi-targeted siRNA against conserved genomic regions of flu: new therapeutics with broad activity against emerging flu strains

Project final report

Executive summary of the final project report outlines the major results achieved throughout the project performance. Detailed results and discussions have been presented in the eight quarterly reports.

Introduction: Among pandemic-prone diseases, viral diseases—specifically influenza—have a significant, if not leading position. The prospect of influenza pandemic generates immediate alarm around the world. Far more contagious than most infections, it is spread by coughing and sneezing, and transmissible within an incubation period too short to allow for contact tracing and isolation. For these reasons, pandemic influenza would have devastating consequences. If a fully transmissible pandemic virus emerged, the spread of the disease could not be prevented. Development of strategies for mitigating the severity of a new influenza pandemic is now a top global public health priority.

Anti-influenza vaccine is a major tool to protect the population or—at minimum— to ease symptoms in infected people. However, anti-flu vaccine is of moderate to low effectiveness for the groups most susceptible to the influenza virus: infants, adults above 65 years of age, and immune compromised people. In addition, the capacity to produce effective anti- influenza vaccine on a timely basis is problematic due to frequent mutations in influenza-causing viruses and the appearance of new viruses. Therefore, the world-class pharmacological strategies for dealing with the influenza pandemic are now based on antiviral drugs.

To date, there are only five well-established anti-influenza drugs commercially available. However, recent studies have demonstrated that even Tamiflu—a champion therapy against influenza—is effective only in about 45-50% cases, and only if administered within the first 24-48 hours post infection. Tamiflu and the other four drugs may reduce the duration of flu symptoms but do not reduce the complications of flu, like pneumonia or hospitalizations, and are not confirmed to reduce transmission of the virus. Moreover, all current anti-flu drugs cause mild to severe side effects. In addition, different subtypes of the influenza virus have already shown the ability to become resistant to existing drugs such as Amantadine, and to the first line state-of-the-art Tamiflu. In fact, H1N1 influenza viruses contain a mutation conferring resistance to Tamiflu—one of the most common resistance mutations seen in treated patients since 2004—which has now circled the globe. This leaves a void in the ability to treat patients at the highest risk.

Among the other anti-influenza drugs currently under development short interfering siRNAs seem to be very hopeful. Ribonucleic acid interference (RNAi) exploits an ancient part of the immune system that protects plants and animals against invaders by the depletion of viral genomic RNA targets in a sequence specific manner by making use of small interfering RNAs (siRNAs)]. Natural mechanism of siRNAs generation within a host cells can be bypassed by the use of synthetic RNA duplexes comprising of 19 nucleotide duplexes. The siRNA duplex comprised of a sense strand homologues to the target and an antisense strand that binds to the target mRNA. One of the major advantages of siRNA-based therapeutics in a pandemic influenza situation is that the design of specific siRNAs only requires knowledge of the circulating viruses' gene sequence, and the siRNAs synthesis can be achieved within a short period of time (as compared to at least six months required for new vaccine production). Although specificity and tissue delivery remain major bottlenecks in the clinical applications of exogenous synthetic RNAi in general, intranasal application of siRNA against respiratory viruses including, but not limited to influenza virus, has experienced significant success and optimism.

To contribute to siRNAs success as new prophylactic and therapeutic remedies, the Project explored the following: 1) new substances to improve siRNAs transfection to target cells; 2) methods for

siRNA administering to improve siRNAs delivery to the target organs; and 3) the "cocktails" designed from siRNAs targeting different genes of influenza virus to achieve antiviral efficacy of siRNAs-based complexes toward multiple influenza viruses and subsequently to increase probability of siRNAs application as prophylactic and therapeutic medicine.

Results Overview: Main accomplishments of the project can be structured in two distinctive sub-sets:

1. In vitro antiviral efficacy of siRNAs

To achieve best possible efficacy of exogenously introduced siRNAs, optimization of siRNA transfection into mammalian cells were performed, because failure to optimize critical transfection parameters can render RNAi effects undetectable in cell culture. These transfection parameters included culture conditions, choice and amount of transfection agent, exposure time of transfection agent to cells, and siRNA quantity and quality.

An ability of several polyelectrolytes (polications) to transport siRNAs duplexes, herein siRNAs, across cell membranes was studied in Madin-Darby Canine Kidney Epithelial cell culture (MDCK). Synthetic polymer Polyallylamine (PAA; 25,000) was shown the most effective polication providing highest siRNA transfection level that was well comparable, or even exceeded the level of siRNAs transfection by commercially available lipofectamine. In addition, positively charged protein P91 and polyethylenimine/polyethyleneglycol (PEI/PEG) have been also used to serve siRNA vehicle across cell membrane; its concentration in the complexes with siRNAs were optimized, and its cytotoxicity was also studied. siRNAs transfection efficacy of the four examined polymers can be arranged in the following order: PAA > Lipofectamine > PEI/PEG > P91.To design optimal polymer/siRNA complexes, the concentrations of the components in the complexes were varied to achieve the best transfection level.

Antiviral properties of several siRNAs complexed with PAA were examined in MDCK culture. Out of nine tested siRNAs duplexes, four siRNAs duplexes, herein coded as siRNA89, siRNA91, siRNA99, and siRNA103, were chosen for further study due to their highest antiviral effect observed during 24 hours. From these four siRNAs, siRNA89 and siRNA103 have been shown the most effective, and therefore, were more extensively studied. It was observed that antiviral effect posed by each of these two siRNAs was dose- dependent. Both, siRNA 89 and siRNA103 reduced viral replication from 4.5 to 1.0 – 1.5 log EID_{50}/0.2ml, correspondingly, at optimal siRNA concentration of 40 nM.

One of the most interesting and important results was received when two siRNAs targeting different genes of influenza virus were applied in combination. Each siRNA was used at concentration of either 20 nM or 40 nM. When the combinations of different siRNAs were used, the total concentration of siRNAs in the complex was 40 nM. Assessment of virus reproduction in the MDCK cells cultivated in the presence of anti-influenza siRNAs complexed with PAA demonstrated that the combination of siRNA89 and siRNA103, posed virus-inhibiting effect that noticeably exceeded the antiviral effect caused by each individual siRNA. Specifically, siRNA89 or siRNA103 at the concentration of 40nM reduced the titer of influenza A virus A/California/07/09 (H1N1)pdm09 from 5.15 to 2.25 - 2.13 logEID_{50}/0.2 mL, correspondingly. At the same time, the combination of siRNAs(89+103) at the total siRNA concentration of 40 nM reduced the infectious titer of the same virus from 5.15 to 1.38 log EID_{50}/0.2 mL. Thus, synergetic antiviral effect of two siRNAs targeting different genes of influenza virus was observed. Fig. 1 illustrates concentration dependence of siRNAs antiviral effect, demonstrates antiviral efficacy of siRNAs complexed with different polymers, and evidences that synergetic effect of siRNAs (89+103) was observed for all polymers used to complex siRNAs.

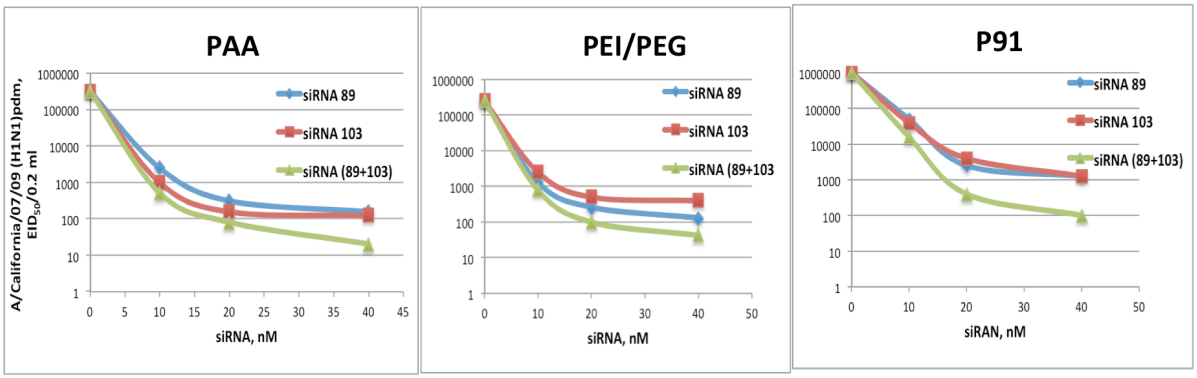

Fig 1. *In vitro* antiviral efficacy of siRNAs complexed with different polymers toward influenza A virus subtype H1N1 as the function of siRNAs concentration and the nature of the polymer.

Synergetic antiviral effect posed by the combination of siRNAs (89+103) to influenza A virus subtype H1N1 was confirmed with the other subtypes of influenza A viruses H3N2 and H5N2 (Fig 2).

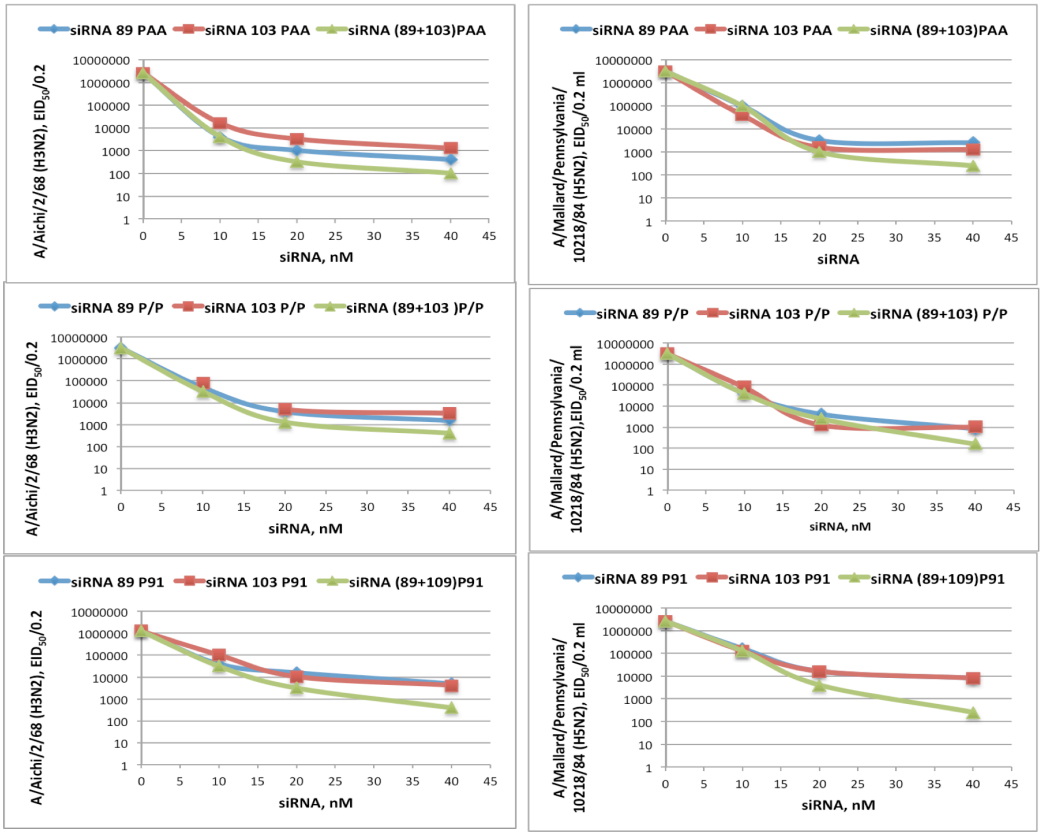

Fig. 2. *In vitro* antiviral efficacy and specificity of siRNAs complexed with different polymers toward influenza A virus subtypes H3N2 (left column) and H5N2) (right column) as the function of siRNAs concentration.

However, *in vitro* introduction of the siRNA91 to the complex siRNAs(89+103) had not increased antiviral efficacy of the complex siRNAs (89+103+91) as compared to antiviral efficacy of the complex siRNA (89+103), despite siRNA91 itself demonstrated high antiviral efficacy and was expected to add efficacy to the complex. No explanation of this fact can be provided at the moment.

2. In vivo administering siRNAs complexes in mice influenza model.

While in vitro experiments with siRNAs provided very important information, the results of these experiments did not allow a critical evaluation of how genes function within the whole organism. To address this question, the most successful results received *in vitro* were used to study siRNAs antiviral efficacy *in vivo*. The success of siRNA mediated gene silencing *in vivo* depends on efficient delivery and retention of the siRNA in the vasculature of a specific tissue of interest, and its effective uptake by those cells. In addition, the siRNA must remain stable until it can reach its ultimate destination.

To develop animal influenza model, the pandemic influenza virus A/California/07/09 (H1N1)pdm09 was adapted to white mice by serial passages in the lungs. It was shown that mice infection with this virus resulted in the reduction of intact cells in bronchial epithelium from 98 to 5%. It was observed that on the third post infection day there was 63% of damaged cells, i.e. more than one half of bronchial epithelium cells at this stage were absent. This model was adopted for *in vivo* siRNAs study.

Several factors influencing antiviral efficacy of siRNAs *in vivo* were studied: different strategies for delivery of siRNAs molecules (Fig 1); application of combine siRNAs targeting different genes of influenza virus versus application of single siRNAs; application of siRNAs complexed with different polymers improving siRNAs transfection across cell membrane; total concentration of siRNA administering to an animal; and a time point after mice infection when an application of siRNAs to infected animals posed an antiviral protective effect.

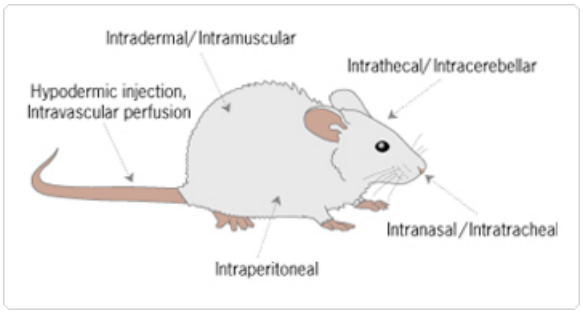

Figure 3. Routs for delivery of siRNAs complexes *in vivo*.

To choose an optimal method for siRNA delivery, fluorescently labeled siRNA was administered intraperitoneally (IP), intranasally (IN), and intratracheally (IT). It was observed that there was no statistically significant difference between the efficacies of applied delivery methods, though, IT and IN delivery methods appeared to be more effective. In addition, it was determined that after IT, IN, and IP administration of fluorescently labeled siRNA, fluorescence was observed in mice kidney, liver, and lungs and was detectable at least 3 hours after siRNA administration. In spite of an importance of these results, their explicit interpretation was not possible because of the biases observed in the experimental protocols applied in this study.

IP administration of siRNAs (89+103)complexed with PAA to infected mice resulted in almost twice reduced mortality among infected mice compared with mice received no treatment, and was equal to that of Tamiflu – golden standard of anti influenza drug (Fig. 2). However, IT administering of siRNA complexes in this subset of the experimnts appeard to be unsuccesful possibly due to high traumatic effect posed by inappropriate device utilized for IT siRNAs administration. Antiviral effect of siRNAs (89 +103)/PAA was shown to be dose- dependent (Fig.

3), and positively depended on the time when siRNAs complx was administered to infected animals. The highest antiviral protective effect was observed when siRNAs(89+103)/PAA was administered 24 hours after animals received the virus (Fig. 4)

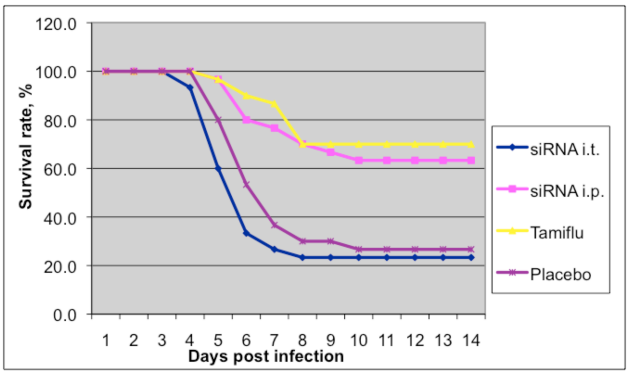

Fig. 3. *In vivo* effect siRNA(89+103)/PAA on the dynamics of animal mortality throughout the duration of the influenza-induced pneumonia caused by the influenza A virus subtype H1N1.

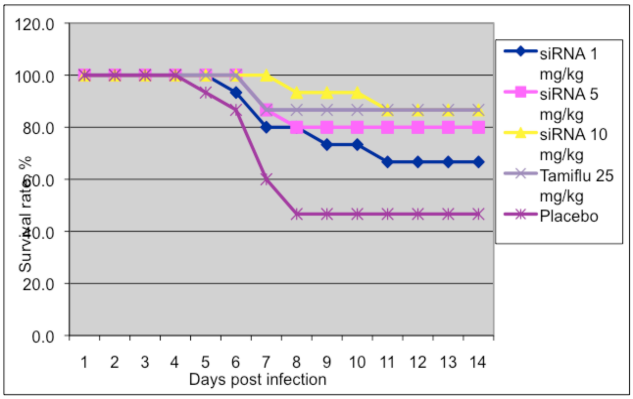

Fig. 4. *In vivo* effect of siRNA(89+103) /PAA dose on the dynamic of animal mortality in the course of the influenza-induced pneumonia, caused by the influenza A virus subtype H1N1.

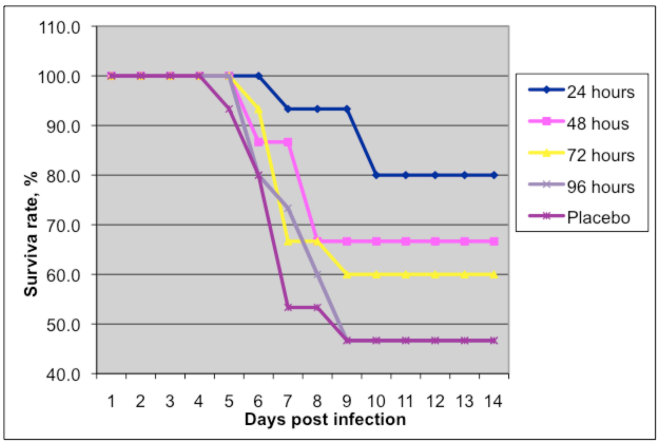

Figure 5. *In vivo* dependence of the dynamic of mice mortality in the course of the influenza-induced pneumonia caused by the influenza A virus subtype H1N1 on the time between the mice inoculation with viruses and siRNA administration.

Antiviral activity of siRNA complexed with different polymers was studied *in vivo* with mice influenza model. Application of siRNA (89+103) complexed with PAA and PEG/PEI caused reliable reduction of virus RNA replication and transcription. Of the two carriers examined PAA seemed to be somewhat more effective. Although *in vivo* administering of both siRNA (89+103) complexed with PAA and with PEG/PEI resulted in reliable reduction of virus replication in mice lung tissue, the siRNA complexes with PAA appeared to be more toxic than the siRNA complexes with PEG/PEI. Severe lung tissue damage was observed in mice received caused high doses of PAA/siRNAs complexes. This included destruction of air-blood barrier with further lung consolidation. In the same time, application of identical doses of PEG/PEI/siRNAs complexes resulted in moderate reactive changes in lung tissue that included slight edema and cell infiltration.

To compliment *in vitro* study, individual siRNAs and the combination of siRNAs targeting different viral genes were complexed with PEI/PEG, and their antiviral efficacy was examined *in vivo* in influenza mice model. Particularly, non-silencing siRNA, siRNA89 and siRNA103, and the combination of two siRNAs (89+103) were tested. For this study mouse-adapted influenza viruses A/Puerto Rico/8/34 (H1N1), A/Aichi/2/68 (H3N2), and A/Mallard/Pennsylvania/10218/84 (H5N3) were used. siRNAs complexes and antiviral golden standard drug -Tamiflu were administered to infected mice intranasally, once a day during three consecutive days. In the control groups of mice phosphate buffer saline (PBS) and non-silencing siRNA (NSsiRNA) were administered to mice instead of siRNAs targeting different virus genes.

It was observed that infecting of the mice with each of three mouse-adapted virus followed by daily intranasal application of sterile PBS led to a development of lethal pathological process with 50-60% mortality.

Among the siRNAs used, application of single siRNA 89 appeared to be slightly more effective in mice protection than that of siRN103. Despite this difference was not statistically significant, it was confirmed by the results that demonstrated that siRNA89 was more effective in suppressing virus replication in the lung tissue of infected mice as compared to siRNA103. This effect was observed for every virus used in the experiment. In general, protective efficacy of siRNA89 and siRNA103 observed in these experiments appeared to be lower than it was expected based on the results received *in vitro*. To explain this fact, we have speculated that negative effect caused by intranasal instillation of siRNAs complexes interfered with therapeutic effect of siRNAs.

Nevertheless, the application of the combination of siRNAs (89+103) resulted in reliable protection of the infected mice (index of protection 33-40%) and corresponding mortality reduction for all viruses tested. Thus, synergetic antiviral effect of two siRNAs targeting different virus genes was demonstrated in mice influenza mode *in vivo* and confirmed the results received *in vitro.*

In the same time, *in vivo* protective antiviral effect of orally administered Tamiflu significantly exceeded that of siRNAs(89+103) administered intranasally, and resulted in 100% survival of infected mice. It remains unclear why in *in vitro* experiments discussed above antiviral efficacy of siRNA(89+103) was equal to antiviral effect posed by Tamiflu, while in *in vivo* experiments antiviral efficacy of Tamiflu significantly exceeded antiviral efficacy of siRNAs(89+103). It could be speculated that Tamiflu was less effective toward mice-adapted influenza virus A/California/07/09 (H1N1)pdm09 used in *in vitro* experiments compared to the mice-adapted influenza viruses A/Puerto Rico/8/34 (H1N1), A/Aichi/2/68 (H3N2), and A/Mallard/Pennsylvania/10218/84 (H5N3) used in *in vivo* experiments. Also, it can be proposed that oral rout of Tamiflu administration was more effective than intranasal delivery of siRNAs in *in vivo* experiments. Recently, negative effect of intranasal application of medicine was revealed in different laboratories. It was shown that intranasal application of liquid preparations worsened pneumonia symptoms in influenza-infected animals.

Non-silencing siRNA either had no effect, or resulted in aggravation of influenza pneumonia and in increased mortality for 17-20%. Particularly in animals infected with H3N2 and H5N3 viruses.

Toxicity of siRNA preparations *in vivo* was examined using intranasal administering. No mortality was observed in the group of non-infected mice received 600 μg of siRNAs/mouse. Thus, 50% lethal dose of the siRNA89 and siRN103 exceeds 30 mg siRNA/kg.

The efficacies of intranasal and intratracheal applications were compared in *in vivo* model study using uranin, an alkaline salt of fluorescein. For intratracheal uranin application Micro Sprayer aerozolizer (PennCentury, USA) was utilized. After uranin administering, the fluorescence in the blood and bronchoalveolar lavage (BAL) was analyzed. The fluorescence observed in serum after intratracheal application of uranin was 2-4 times higher than that after intranasal administration. In BAL, fluorescence decreased 3 times during 30 min after intranasal administration of uranin. Contrary to that, fluorescence has not change during 30 min after intratracheal application and was 120-500 times higher as compared to that of intranasal uranin delivery.

Intratracheal administering of siRNA complexes was shown more effective as compared to intranasal siRNAs administering ((index of protection = 50% versus index of protection =16.7%, correspondingly). Whether or not this difference was associated with negative effect of intranasal application (see above) remains unclear.

Conclusion: The project was performed in very close collaboration between the scientists from the Institute of Influenza (RII), the Institute of Highly Pure Biopreparation (IHPBP), General Research Laboratory Inc. (GRL), and Pacific Northwest National Laboratory (PNNL). The role of each partner was indispensible in successfull completion of the project.

It has to be emphasized that the project was inspired by GRL Inc. research team, which suggested the scope of work, generously shared their experience in siRNAs field with the project participants, designed siRNAs molecules for the project, and supplied the project participants with siRNAs and the other reagents throughout the project. In addition, GRL project PI played proactive role in the results discussions and protocols development. Highly experienced research team from RII and IHPBP fully complemented the project by conducting all experimental work, contributing their expertise in virology and antiviral drags design, and creativity in experimental set-up. PNNL supported the project by data analysis, contributed to experiments planning, results compilation, analysis and interpretation.

The main project outcomes are the following:

1. We identified several siRNAs against select Flu gene segments that showed efficacy in silencing the flu genes
2. Combining these siRNAs in various combinations we identified that siRNAs(89+103) or siRNAs(89+105) were very potent at inhibiting the flu viruses
3. We observed that while administration of siRNAs(89+103) intranasally was not as efficacious as its intraperitoneal administration, an additive (cooperative/synergetic) effect of combined siRNAs was characteristic for both, intraperitoneal and intranasal siRNAs delivery.
4. siRNAs complexed with PAA administered intraperitoneally posed higher antiviral effect than that of siRNAs complexes with PEG-PEI.
5. Through GRL own bioinformatics it was identified that siRNAs (89+105) targets the emerging influenza A virus subtype H7N9 VERY effectively compared with siRNAs (89+103)
6. Based on GRL earlier bioinformatics approach the combinations of siRNAs(89+103) or siRNAs (89+105) targets more than 99% of all known flu strains.

Overall, it is believed that the project results made very significant contribution to alternative flu therapeutic that has the ability to target multiple flu strains, and this therapeutic could be used:

a) To treat the elderly and infants with flu who do not produce a good therapeutic effect to vaccines;
b) To treat newly emerging strains of flu for which there are no vaccines

In addition, it is proposed that:

c) (Unlike Tamiflu), siRNAs can be administered more than 24hrs post infection and still show efficacy
d) Can be applied through IP or IN routes.
e) May not need to be reformulated every time a new flu virus emerges
f) Demonstrates the general concept of using siRNAs to treat rapidly emerging viral threats.

Prospected publications and presentations

The main finding of the project will be submitted to Nature Biotechnology - one of the most prestigious journals in this field. The draft of the manuscript is in the progress. RII team is expected to provide some additional results to complete the manuscript. This manuscript will have multinational authors, including from GRL and PNNL, US; RII/IHPBP, Russia; and Chinese specialists who contributed their results with dangerous H7N9 Flu strain recently emerged in China. After the main manuscript is accepted for publication, the results of the project will be presented on relevant Worshops and Conferences, and smaller articles will be additionally published.

Path forward and prospected commercialization:

GRL Inc., remains actively engaged with RII team and they continued to work together after the project completion. GRL Inc. is actively seeking external (VC or Government) funding to move siRNAs-based therapeutic ahead. This funding will be used to support RII and GRL joint activities.

GRL Inc. is planning to support siRNAs clinical investigation in Russian Federation, particularly in RII, which is the WHO Center for Influenza in Russian Federation. Academician Oleg Kiselev, who is WHO member and the Director of RII/WHO Center, is seeking a complimentary funding from WHO and the Russian Ministry of Health, both oversee RII.

RII/IHPBP put together the set of the documents that need to be submitted to the Ministry of Health of Russian Federation to approve siRNAs-based therapeutic for Phase I Clinical trial.

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