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### Title and Subtitle
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### 14. Abstract
The objective of this project is to develop novel formulation strategies for delivery of Flufrivitide-3 and other therapeutic peptides. Therapeutic peptides can be delivered in a non-invasive manner through the nasal mucosa and through the lungs. A wide range of factors, including mode of inhalation and particle size, influence the deposition of aerosols within the respiratory tract. Chemical modification of the Flufrivitide-3 will be evaluated to optimize its functional activity. The use of micro- and nano-technology will also be explored through the fabrication of particles encapsulating the peptide that are specifically suited for nasal and pulmonary delivery. The micro- and nano-particle carriers to be considered include a dry powder formulation, microemulsions, nonspherical liposomes, ceramic shell vesicles, and nanometer-sized silk particles. Nasal administration of soluble Flufrivitide-3 both pre- and post-exposure to influenza virus has been shown to be effective in preventing infection in an in vivo animal model. However, multiple doses, pre- and post-exposure were required for efficacy. We hypothesize that the proposed techniques will enhance the efficacy of the therapeutic peptide itself, thereby reducing the required dose, number of doses, and thus the cost of treatment, and improve distribution and release within the upper respiratory tract, thus expanding the duration of bioavailability and efficiency of peptide delivery.

### 15. Subject Terms
Therapeutic Peptides, Bioterrorism, Biothreat, Nanocarrier, Immunity, Prophylaxis
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INTRODUCTION

Influenza virus infections are among the most prevalent of all viral infections in man with between 10% and 20% of the United States’ population suffering from influenza each year. Most individuals recover from influenza in one to two weeks. However, the very young, the elderly and persons with chronic medical conditions can develop post-flu pneumonia and other fatal complications. On average, more than 100,000 people in the United States per year are hospitalized as a result of influenza, with more than 35,000 deaths.

It is possible for the genomic segments of Influenza virus to mix and reassort in co-infected cells to produce a new virus with most segments from the human virus, but with segments encoding the HA and/or NA from the virus of a different animal species (i.e., avian, porcine). Because it contains gene segments encoding proteins for effective replication in humans, the reassorted virus may possess the ability to spread from person-to-person, but would have surface proteins to which people had not been previously exposed. Thus, most individuals will have little or no immune protection against the reassorted virus. The currently circulating strain of avian influenza is an H5N1 strain capable of directly infecting humans and other mammals, resulting in a mortality rate that exceeds 60% among nearly 400 confirmed human cases. This new H5N1 avian influenza A virus is of great concern because it possesses some potential for human-to-human transmission. A novel influenza A (H1N1) emerged in April 2009, and that strain also demonstrated efficient person-to-person transmission. CDC estimates that there were between 43 million and 89 million cases of 2009 H1N1, between 195,000 and 403,000 H1N1-related hospitalizations, and between 8,870 and 18,300 2009 H1N1-related deaths in the U.S. during the period April 2009 and April 10, 2010. Clearly, both seasonal influenza and novel reassortments have the ability to impact both public health and military readiness.

Because of the uncertainty that an antigenically-matched protective vaccine can be made rapidly available, antiviral drugs offer the best method to prevent the spread of a newly emerging strain of influenza A virus in the early stage of a pandemic or after intentional release. Only two classes of drugs are currently available for influenza, adamantanes and NA inhibitors (NAI, oseltamivir and zanamivir). Drug-resistant variants of both of these drug classes have been shown to arise in both seasonal and pandemic strains of influenza viruses, including the novel influenza A (H1N1).

We have recently identified a candidate anti-viral therapeutic peptide, designated Flufirvitide-3. We found that Flufirvitide-3 is a potent inhibitor of infections by divergent influenza viruses in vitro, including H1, H3 and H5 subtypes and influenza B viruses. When administered to the nasal cavities of ferrets, Flufirvitide-3 effectively blocked development of influenza. Flufirvitide-3 and other peptide inhibitors of viral fusion have a number of potential applications including pre- and post-exposure prophylaxis against seasonal or pandemic influenza.

This application is organized into four Specific Aims which will constitute the Technical Objectives (and Milestones) of the proposal. (1) Synthesize modified versions of Flufirvitide-3 peptide through chemical conjugation of specific functional groups to the peptide and synthesis of multimers of the active peptide. (2) Formulate the following micro- and nano-carriers for encapsulation of active peptide and suitable for pulmonary delivery: dry powder formulation, microemulsions, nonspherical liposomes, ceramic shell vesicles, and nanometer-sized silk particles. (3) Confirm the conformational stability of the chemically modified peptide constructs and the structural stability of the delivery vehicles over extended time periods. (4) Evaluate the effect of encapsulation and chemical modification of Flufirvitide-3 on viral inhibition using an in vitro immunoplaque assay.

BODY

The objective of this project is to develop novel formulation strategies for delivery of Flufirvitide-3 and other therapeutic peptides. Therapeutic peptides can be delivered in a non-invasive manner through the nasal mucosa and through the lungs. A wide range of factors, including
mode of inhalation and particle size, influence the deposition of aerosols within the respiratory tract. Chemical modification of the Flufirvitide-3 will be evaluated to optimize its functional activity. The use of micro- and nano-technology will also be explored through the fabrication of particles encapsulating the peptide that are specifically suited for nasal and pulmonary delivery. The micro- and nano-particle carriers to be considered include a dry powder formulation, microemulsions, nonspherical liposomes, ceramic shell vesicles, and nanometer-sized silk particles. Nasal administration of soluble Flufirvitide-3 both pre- and post-exposure to influenza virus has shown to be effective in preventing infection in an in vivo animal model. However, multiple doses, pre- and post-exposure were required for efficacy. We hypothesize that the proposed techniques will enhance the efficacy of the therapeutic peptide itself, thereby reducing the required dose, number of doses, and thus the cost of treatment, and improve distribution and release within the upper respiratory tract, thus expanding the duration of bioavailability and efficiency of peptide delivery.

In the first funding cycle, we explored three different approaches: 1) N-terminal modification of Flufirvitide-3 via conjugation of an NHS-ester modified 4-azido butanoic acid, 2) Polymeric modification of Flufirvitide-3 - PCL to investigate increasing hydrophobicity, PEG to investigate increasing hydrophilicity, and a variety of multiarm cores to investigate synergic effect of multiple peptides that are covalently tethered together, and 3) Analysis of different excipients for stable lyophilization and dry powder pulmonary delivery.

In the second funding cycle, we 1) optimized the conjugation of Flufirvitide-3 to PEG, 2) built a diverse library of azide functionalized polymers, 3) developed novel formulations for parenteral and mucosal delivery of Flufirvitide-3 and other peptides, and 4) prepared additional Flufirvitide-3 lyophilized powder formulations for pulmonary delivery.

In the last funding cycle, we transitioned to Specific Aim 4: Evaluate the effect of encapsulation and chemical modification of Flufirvitide-3 on viral inhibition using an in vitro immunoplaque assay. Below is an example of one of the microemulsions we have developed for this Specific Aim.

**Flufirvitide-3 Microemulsion Formulation**

These microemulsions are oil-in-water (O/W) type, with thermodynamically stable, transparent and low viscous properties. Since Flufirvitide-3 has hydrophobic and hydrophilic regions, high local administration concentration is expected due to the adsorption of Flufirvitide-3 on the interface of O/W interface, facilitating efficient administration.

These O/W microemulsions are composed of an aqueous phase (PBS buffer, pH=7.4), oil phase (isopropyl myristate, IPM), surfactant (polysorbate 80, Tween 80) and cosurfactant (propylene glycol, PG). During the fabrication of these microemulsions, the surfactant Tween 80 and cosurfactant PG were weighed at the ratio of 4:1 (w/w), and vortexed vigorously to make the surfactant mixture (S mix). Surfactant mixture was then mixed with the oil phase IPM, and the resulting mixture was finally vortexed with the required amount of PBS buffer. Samples were kept at room temperature to equilibrate overnight. Clear and isotropic samples are determined to be within the oil-in-water microemulsion region.

The pseudo-ternary phase diagram, as shown in Figure 1, was constructed for PBS buffer, IPM oil and surfactant mixture to determine the microemulsion region. In Figure 1, the region to the right of the boundary line is transparent.
O/W microemulsions with lower viscosity. The final formulation identified to use in our experiments contained Flufirvitide-3 in PBS buffer as aqueous phase, surfactant mixture and IMP oil phase at the ratio of 6:4:0.3 respectively, with the final concentration of Flufirvitide-3 at 10 mM. Such formulation will be used in our further studies.

Figure 2. A: visual observation of O/W microemulsions with the compositions of Flufirvitide 3 aqueous solution, surfactant mixture and IMP oil phase at the ratio of 6:4:0.3; B: cryo-TEM image of O/W microemulsions.

We have begun evaluation of the effect of encapsulation of Flufirvitide-3 within phospholipid liposomal vesicles and are currently in the process of having the modified Flufirvitide-3 synthesized in sufficient quantity/purity for evaluation.

In order to confirm that the Flufirvitide-3 peptide and derivatives maintain activity through the various formulation processes and chemical modification techniques, in vitro immunoplaque assays will be performed. Influenza A/H1N1 will be used since we have previously demonstrated the effectiveness of Flufirvitide-3 against this strain. In a typical assay, virus is pretreated with Flufirvitide-3 for one hour and subsequently used to infect a cell monolayer. One-hour after infection of the monolayer, the virus inoculum is removed and a low-viscosity Avicel overlay media with or without active peptide is added to cover the monolayer. After incubation, the cells are fixed and stained to detect influenza virus nucleoprotein and indicate viral plaque formation.

Shown below is a representative result. Untreated virus was added to the first two wells for infection of MDCK cells. In the last two wells, virus was incubated with 10 µM Flufirvitide-3 for 1 hour prior to infection of the cell monolayer. As seen below, we observed a reduction in the number and the size of viral plaques when virus was incubated with Flufirvitide-3 prior to infection of cell monolayers. This resulted in an 86.25% reduction in viral PFUs.

This assay will allow us to compare the activity of Flufirvitide-3 in its native form (above) with the various chemically-modified conformations obtained in years 1 and 2 to identify those exhibiting enhanced antiviral activity. Pretreatment of the cell monolayer with our different micro- and nano-carrier formulations for various time points in addition to extended incubation post-infection will also be included. This will allow us to assess whether the extended release of
peptide from these specifically-tailored vehicles will provide sustained release of active protein to increase protection of the monolayer pre- and post-infection.

KEY RESEARCH ACCOMPLISHMENTS

- Encapsulation of Flufirvitide-3 in microemulsions
- Established an in vitro immunoplaque using Influenza A/H1N1 and demonstrated the efficacy of Flufirvitide-3 in this model.

REPORTABLE OUTCOMES

None to date.

CONCLUSIONS

Flufirvitide-3 and other peptide inhibitors of viral fusion have a number of potential applications:

- be used for pre- or post-exposure prophylaxis in civilians of all ages and in military personnel, including first responders, for seasonal or pandemic influenza viruses or to protect from deliberate release of a new strain of influenza virus.
- be used for treatment of persons of all ages infected with seasonal or pandemic influenza viruses.
- be stock-piled in the event of an emerging pandemic strain of influenza A virus in a stable formulation, with use as pre- or post-exposure treatment and prophylaxis.
- be used to protect personnel working in biodefense laboratories from accidental exposures to highly pathogenic influenza viruses.
- be used to protect persons traveling to locales where influenza is endemic from exposure to novel strains of influenza virus.
- be used to protect hospital personnel from exposure to influenza virus from infected patients.
- serve as a deterrent from using this easily acquired pathogen as a bioweapon.
- pilot a platform technology, which may be useful against other viruses that are potential bioweapons.

Nasal administration of soluble Flufirvitide-3 both pre- and post-exposure to influenza virus has been shown to be effective in preventing infection in an in vivo animal model. However, multiple doses, pre- and post-exposure were required for efficacy. We hypothesize that the proposed techniques will enhance the efficacy of the therapeutic peptide itself, thereby reducing the required dose, number of doses, and thus the cost of treatment, and improve distribution and release within the upper respiratory tract, thus expanding the duration of bioavailability and efficiency of peptide delivery.