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Development of Glucan Particle Encapsulated Nevirapine as a Topical HIV Microbicide

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Development of Glucan Particle Encapsulated Nevirapine as a Topical HIV Microbicide

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ABSTRACT

HIV is a highly mutable virus that has become a global pandemic. In order to prevent further spread of this disease a topical microbicide that is cheap and easy to administer and produce is necessary. Our project involved the extraction of the HIV drug Nevirapine from commercial tablets to encapsulate it inside glucan particles for mucosal delivery. Glucan Particles (GP) are naturally internalized by macrophages and Langerhans cells thought to be the first cells to be HIV infected at mucosal sites. GP are hollow microparticles derived from baker's yeast that can be used to absorb the Nevirapine into an encapsulated core. To keep the drug trapped inside the core various synthetic strategies were evaluated to identify an effective formulation for future *in vitro* and *in vivo* testing.

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BACKGROUND

Since 1981, acquired immunodeficiency syndrome (AIDS) has been a pandemic affecting millions of people in countries all over the world. Through years of research, scientists now know that "AIDS is caused by infection with a virus called human immunodeficiency virus (HIV)" (CDC). HIV is a lentivirus, which is part of a larger group of viruses called retroviruses. Lentiviruses are categorized by the fact that they are slow to fully infect and thus take a long time to produce lasting negative side effects in the body. HIV interacts with the primary cell surface receptor CD4+ to gain entry into white T cells. These cells are crucial to fighting off infections within the body, and when they are destroyed, the body is left vulnerable to other infections and diseases. A person infected with HIV can carry the disease and never develop any serious side effects until the number of T cells with CD4+ receptors in their body has greatly decreased. HIV is a very dangerous virus due to the fact that it can mutate readily, and one person can be infected with different strains of HIV at the same time. HIV can only replicate within cells, and it directly attacks the immune system. Worldwide at the end of 2008, there were a reported 33.4 million people living with HIV/AIDS, and since 1981 more than 25 million people have died from this virus.

Origin of HIV

HIV is thought to be originally derived from Simian Immunodeficiency Virus (SIV) based on the discovery that many strains of SIV have a very strong resemblance to known HIV strains. SIV affects monkeys and it is believed to have crossed species through zoonosis, which is a viral transfer between humans and animals. This transfer could have occurred if a human had consumed an infected monkey or had blood transferred from an infected animal into an open cut

or wound. The exact means of zoonotic transmission is unknown, but there are many theories circulating as to how humans became infected with this virus. Nevertheless, researchers have concluded that the virus originated in West Central Africa between 1915 and 1940 (Korber et al., 2000).

Types of HIV

The two known types of HIV are HIV-1, which is the version of the virus that is predominantly known worldwide, and HIV-2, which is uncommon except in West Africa. Although HIV-2 is less easily transmitted, both can be contracted through sexual contact, blood transfusion, or from mother to child during pregnancy, childbirth or breastfeeding. Both HIV viruses are believed to cause AIDS, and after infection the two types are virtually indistinguishable.

HIV-1 is categorized into four groups and these groups are believed to represent four separate transmissions of SIV to humans (**Figure 1**). The major type of HIV-1 is group M, and this group is then broken down into 9 subgroups. These subgroups are all genetically distinct from one another, and can even recombine together to create new hybrid viruses. When two subgroups mix and can be seen in more than one person they are known as Circulating Recombinant Forms (CRFs) of the virus.

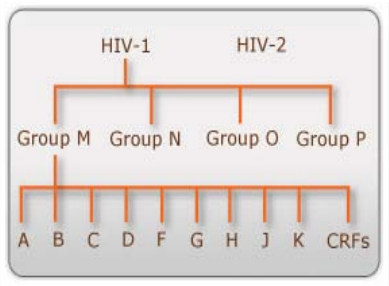

Figure 1: Different Levels of HIV Classification *(*"HIV types, subtypes, groups & strains", *2010)*

Worldwide, the different subtypes are very unevenly distributed, but the most widespread of all the types are A and C. Subtypes A and C are most commonly seen in Africa and India. Type C has also caused the worst epidemics and is responsible for nearly half of all HIV infections worldwide. Subtype B is the most common form of the virus found in the Americas and in Europe *(*"HIV types, subtypes, groups & strains, 2010*).* It is believed by scientists that more combinations and mutations of the disease will continue to appear and that the different subtypes will spread to new areas globally as the HIV epidemic continues. If this happens it will make preventing and possibly stopping the spread of HIV much more difficult.

HIV-1

As stated before, HIV needs to infect cells within a living organism in order to start the transcription process to replicate itself. Outside of a cell, an HIV particle measures about 0.1 microns. In comparison to the CD4+ white blood cells that it infects, an HIV particle has one seventieth the diameter and is so small that it can only be imaged with an electron microscope.

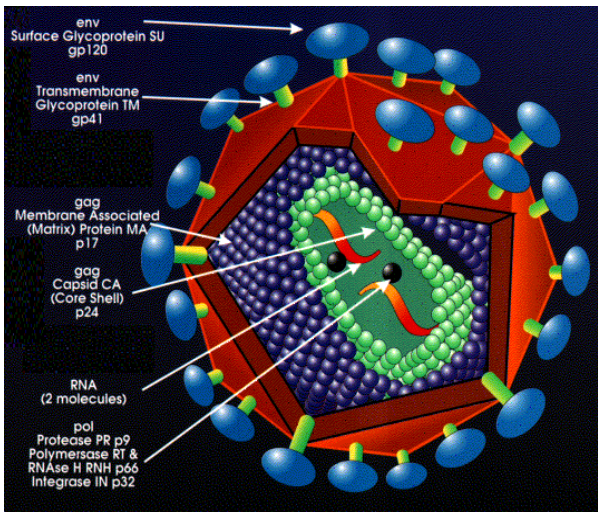

Figure 2: Diagram of the HIV Virus (Brinkhof, 2010)

A viral envelope, a matrix and a viral core are the three elements that make up the basic structure of an HIV particle (**Figure 2**). Each of these components are made of specific proteins, and these are integral to the way a particle is able to function and protect itself in the human body. The viral core is at the center of an HIV particle and is also known as a capsid. The capsid contains two copies of positive RNA that code for the nine genes associated with HIV. This core also contains a pol gene which consists of reverse transcriptase, integrase and protease, which are the three factors necessary for HIV replication. The matrix surrounds the viral core and is responsible for maintaining the stability of the virus. The protein that makes up the capsid is p24, and the matrix protein is p17, and both of these are part of the Gag polyprotein which provides the basic physical structure of the virus. The viral envelope is the outer layer of the HIV particle, and from this layer complex HIV proteins protrude to form what appear to be spikes on the surface of the particles. These protein spikes are made from gp120 and gp41. The other parts of HIV's genome are three transctivators and three regulators (**Figure 3**). The transctivators are Tat, Rev, and Vpr, which work to enhance gene expression by increasing levels of transcription, and giving HIV a structure that can more easily overtake its host's defenses. The regulators are Vif, Nef and Vpu which disrupt antiviral activity, allow replication of the virus, and enhance virion release. (Jia, 2010).

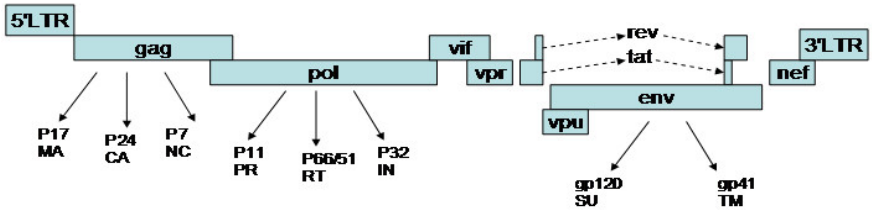

Figure 3: Diagram of the HIV Genome. (Costin, 2007)

HIV infects CD4+ cells by using these glycoprotein spikes to bind to the CD4 and fuse to the cell membrane. Once fusion occurs, the particle releases its contents into the cell where the HIV enzyme reverse transcriptase converts viral RNA to DNA. The DNA formed is then spliced into the human DNA by integrase which is another HIV enzyme and becomes proviral DNA. When a cell that contains a provirus is activated, the human body treats the HIV infected genes the same as normal genes, and converts the DNA to mRNA which is then transported out of the cell and used as a blueprint to continue producing more HIV. The strands of mRNA contain complete copies of HIV genetic material, and long strands are hydrolyzed by protease to form shorter pieces which are all able to form more mature viral cores. Once this begins occurring in the body, more and more cells are infected, and unless HIV is diagnosed the immune system will continue to become more compromised.

Two cells particularly important in HIV transmission are macrophages and Langerhans cells. Both types of cells are the initial targets of HIV and provide an efficient way for HIV to access T cells. Macrophages are large cells derived from white blood cells that are found in many different types of tissue. Macrophages work by digesting foreign microorganisms and damaged cells. It has actually been stated that macrophages "form a reservoir of HIV-1 in infected persons" (Groot et al., 2008). HIV can be stored in macrophages because the virus assembles itself into a sort of intracellular compartment, and in some instances this protects the virus from being attacked by antibodies. Langerhans cells are dendritic cells that that are located in epithelia. Langerhans cells characteristically cluster with numerous different types of T cells and this is thought to be the main reason explaining why such a small amount of HIV can quickly replicate enough to infect a whole immune system. The site where Langerhans cells and T cells interact creates the perfect atmosphere for explosive and rapid HIV production. HIV is

thought to move from mucosal tissue to lymph nodes through transmission by Langerhans cells as well. Both of these cells are key reasons why HIV is able to access T cells which, in turn drastically decreases the efficiency of the immune system against infection. Based on this knowledge, it seems that these are areas that it may be beneficial to halt future HIV infections.

HIV Medications

The main treatment option after infection with HIV is antiretroviral drug treatment. Advances in this area have allowed for infected individuals to maintain fairly normal lives as long as the HIV drugs are taken every day. Antiretroviral drugs work to keep the amount of HIV in the body at a minimal level, which in turn decreases the amount of damage and slows the weakening of the immune system. Although the drugs work to prevent HIV damage, the exact time to start treatment is debated due to adverse side effects of the drugs. Generally it is believed that treatment should not begin until the later stages of HIV infection. A CD4 test measures the number of helper T cells in a sample of blood. A person uninfected with HIV will normally have between 500 and 1200 cells/mm^3. Since HIV decreases the amount of CD4 in the blood, guidelines have been set up to determine exactly what levels of CD4 are considered dangerous enough to start treatment. It is recommended that antiretroviral treatment being once an infected individual reaches a level of CD4 under 350.

There are five groups of antiretroviral medications and each is classified by the way they attack HIV. Since HIV can mutate so easily treatment of HIV usually consists of combination therapy which involves taking more than one drug at a time. Combination therapy largely reduces the rate of HIV resistance to the drugs. The two most common groups of HIV drugs are Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and Non-Nucleoside Nucleoside Reverse

Transcriptase Inhibitors (NNRTIs), and both of these are available in most countries. Both NRTIs and NNRTIs interfere with reverse transcriptase which inhibits HIV from replicating within the cells. There are many combinations of drugs but both the World Health Organization, and American guidelines recommend the use of at least one NNRTI in treatment. The WHO states that the first line of treatment should be one NNRTI and 2 NRTIs. American guidelines recommend one NNRTI or a protease inhibitor in combination with two NRTIs. The other types of medication include Protease Inhibitors, Fusion Inhibitors and Co-Receptor Inhibitors. All of these interact with HIV in a way that limits interaction between the virus and healthy cells. These also are able to interfere with the conformation of the virus which slows down the rate it can infect healthy cells.

Nevirapine

One NNRTI that has been on the market since 1996 is Nevirapine. This drug was developed by Boehringer Ingelheim Pharmaceuticals, Inc. (Hargrave et al., 1991). Nevirapine was the first NNRTI approved by the FDA, and is considered a breakthrough in HIV treatment. This drug works to lower the viral load, or the amount of HIV, that is present in the blood. Like other NNRTIs, Nevirapine attaches itself to the reverse transcriptase and hinders the virus' ability to reproduce. Specifically the drug binds to heterodimeric HIV-1 reverse transcriptase and disrupts the catalytic site of the enzyme. Nevirapine also prevents viral RNA from being changed to DNA which also halts replication. By limiting the amount of HIV in the blood, this drug works to maintain the amount of CD4 cells in the body. Studies on Nevirapine have shown that 90% of Nevirapine taken orally is absorbed into the body, and after initial absorption the drug is widely distributed to nearly every tissue in the body. Nevirapine must be taken in combination

with other medications in order to ensure it usefulness. It is not fully known how HIV-1 eventually becomes resistant to Nevirapine, but it is believed that a single mutation of the reverse transcriptase may be adequate for HIV to develop a high level of resistance. Also Nevirapine has no known effects against HIV-2.

Albumin Binding Capabilities

HSA or human serum albumin is produced in the liver, and is the most abundant protein in blood plasma. It is known for its ability to bind to ligands, which includes some drugs. Due to this knowledge, HSA was tested and now there is conclusive evidence to prove that HSA can actually bind to certain HIV medications. There is "clear cut evidence for the allosteric inhibition of anti-HIV drug binding to HSA." HSA is made of single non-glycosylated chains that contain three homologous domains. The structural organization of the protein is what creates all the binding sites for ligands.

Although the combination therapy offered from many anti-HIV drugs has improved mortality rates, there is potential for a new way to administer these drugs. "One of the most important factors affecting the distribution and the free, active concentration of many administered drugs is binding affinity for HSA" (Fanalli et al., 2007). The interactions of plasma proteins with anti-HIV drugs can drastically increase the efficiency of these drugs. It has been discovered that at concentrations used in therapy, Nevirapine binds to HSA and increases the affinity of heme, which is the site of oxygen binding, for HSA. Since Nevirapine is "a small hydrophobic butterfly-shaped ligand" (Fanalli et al., 2007) its size doesn't limit its preferential binding capabilities.

Studies of the binding ability of different anti-HIV drugs to plasma proteins have shown that Nevirapine binds with ~70% efficiency to Human serum albumin (HSA). Proteins like HSA that are present in plasma have strong ligand binding capacity to different groups. HSA binds drugs non-specifically or by interaction at any of seven binding sites with different affinity. In the case of Nevirapine, it has been found that it binds effectively to the FA7 site, which has been identified as a preferential binding site for bulky, heterocyclic molecules (Fanali et al., 2007). In order to prove that Nevirapine could bind to FA7, which is also known as Sudlow's site I, a docking analysis was performed. Nevirapine has the potential to act as an allosteric effector and this characteristic allows the Nevirapine to easily bind to HSA.

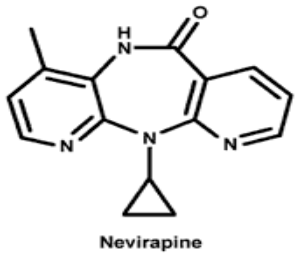

Figure 4: Structure of Nevirapine

AIDS Vaccines and HIV Microbicides

Due to the high level of variance between each of the strains of HIV, the development of any type of vaccine has proven to be quite challenging. Other challenges affecting the progress of vaccine development include a lack of knowledge on exactly how to protect the immune system from the virus, as well as a lack of relevant animal models that would predict similar responses of a vaccine in humans. Due to these factors, scientists have also been trying to develop HIV microbicides, which are antimicrobial products that would prevent HIV transmission through sexual contact. In order for microbicides to work, they would employ mechanisms that either kill or inactivate pathogens, strengthen the body's defenses, block

infection by creating a barrier between the pathogen and target cells, or by preventing infection from spreading from infected cells to healthy ones (Microbicides, 2010) (**Figure 5**).

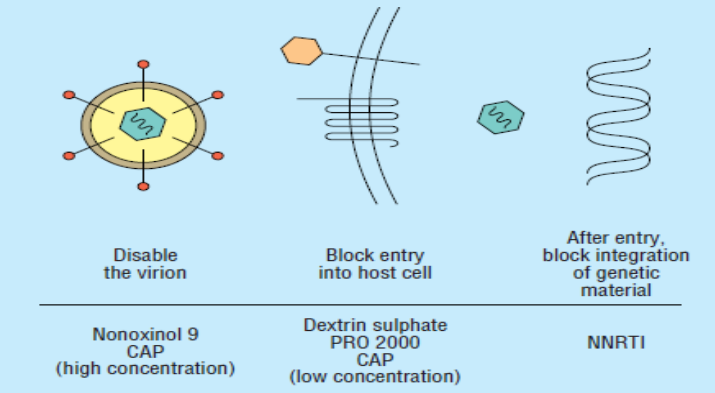

Figure 5: Depictions of the Ways HIV Medications Halt HIV Replication (McCormack, 2001)

Another benefit of an HIV microbicide is the potential to make it regularly available to the public. It is important to remember that "a microbicide will not be a 'silver bullet' for ending the epidemic, but rather another tool to add to existing prevention efforts"(Avert, 2010). Still, it is important to continue to search for any method whether it is a vaccine or microbicide that will be able to lower the rate the HIV pandemic is spreading.

The Glucan Particle Delivery System

Research has proven that "an important aspect of any new drug or vaccine formulation is a component that enhances its safety and efficacy by providing a delivery mechanism and, in the case of vaccines, by boosting the immune response to the antigen." (Spiros et al., 1991). β-Glucans have been referred to as "biological response modifiers," and are known for their ability to activate the immune system. Applications of materials derived from glucans include the use of glucan particles (GP) for macrophage targeted drug delivery. (Chihara, 1992).

Glucan particles are hollow, porous, 2-4 μm spherical particles extracted from *Saccharomyces cerevisia* (Baker's yeast). In order to prepare the β-Glucans, the yeast cell wall is treated so that the cell wall components and the soluble proteins within the yeast cell are removed (**Figure 6**).

The glucan particles are composed primarily of β-1, 3-D-glucan and small amounts of, residual chitin. Encapsulation of payload molecules within glucan particles allows for protection and targeted delivery of the drug into cells with β-glucan cell surface receptors (dectin-1 and Complement Receptor 3 or CR3) such as macrophages, neutrophils and dendritic cells.(Brown, 2001). This makes the glucan particles an efficient delivery vehicle to target cells in the immune system.

Previous work has focused on developing methods to encapsulate macromolecular drugs (siRNA, DNA, proteins) inside glucan particles based on the "in situ layer-by-layer synthesis of electrostatically bound complexes caged within hollow yeast cell wall particles" as depicted in **Figure 7** (Soto and Ostroff, 2008).

The ability to protect and deliver drugs to macrophages, in addition to the immune boosting properties of glucan, makes glucan particles a suitable option to develop formulations containing HIV drugs as potential microbicides. The medication, in this case Nevirapine, would be packaged into a core inside the particle, and then would release the drugs once it was delivered into the body. The methods for obtaining a core that will not only contain the drug but release it back out is a process that in due time will hopefully be properly developed. In the case of the cores made with Nevirapine. The drug would bind to a protein which would in turn bind to the tRNA. Various formulations using different glucan particle batches as well as different

substances in the core were used in order to accurately determine which ones were the most beneficial in trapping and releasing Nevirapine.

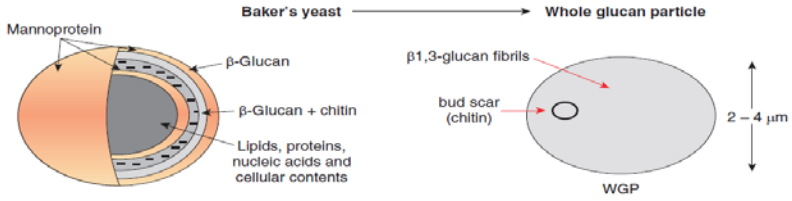

Figure 6: Glucan Particles before and after removal of cell wall components (Yan et al., 2005)

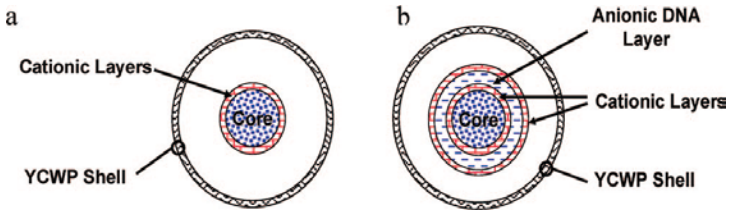

Figure 7: Yeast Glucan Particles Containing Various Cores (Soto and Ostroff, 2008)

PROJECT PURPOSE

The effects of HIV/AIDS are far reaching, and at this point in time it seems that progress is being made but more must be done to ensure that the transmission of HIV is severely decreased. Previous experiments using β-glucan particles have shown success in using this technology as a delivery vehicle for macromolecules (i.e. DNA, siRNA, proteins), and there is current interest to extend its use to delivery of small drug molecules.

The main purpose of this project was to evaluate the use of glucan particles to encapsulate the anti-HIV drug Nevirapine. In order to successfully do this, it was first required that the drug was properly characterized and to evaluate the best conditions for trapping of Nevirapine within protein/tRNA nanoparticulate complexes. After conditions for Nevirapine trapping were identified, several assays were developed to measure the loading of the drug as part of a Nevirapine/protein/tRNA complex inside the glucan particles. After successful loading, the samples were examined in order to determine the release conditions from each GP nevirapine core formulation. If these methods proved to work as expected, the goal would be to make sure the different formulations were not toxic to cells.

MATERIALS AND METHODS

Materials:

Reagents:

Nevirapine (Viramune) 200 mg tablets (Boehringer Ingelheim, Ridgefield, CT).
Ovalbumin (OVA) from chicken egg white, Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), Ribonucleic acid from torula yeast, Type VI (tRNA), 25 kDa branched polyethylenimine (PEI), ethanol, acetic acid, and HPLC grade methanol were purchased from Sigma-Aldrich (Allenstown, PA) and used as received.
Dulbecco's Phosphate buffered saline (PBS) was obtained from Invitrogen (Carlsbad, CA).
0.9% sodium chloride was purchased from Baxter Healthcare Corporation (Deerfield, IL)
Glucan particles were available in Dr. Ostroff's laboratory and were prepared according to previously published procedures (Soto and Ostroff, 2008)
Stock solutions of OVA and BSA at 50 mg/mL in water
Stock solution of Nevirapine at 10 mg/mL in acetic acid pH 2
Stock solution of tRNA 1.5 mg/mL in 0.9% saline

Equipment:

Mortar with pestel,
15 and 50 mL centrifuge tubes and 1.5 mL Eppendorf tubes
Virtis lyophilizer (Virtis Company, Gardiner, NY)
Nanosep 3 k Omega centrifuge filters (Pall Corporation, Ann Arbor, MI)
96-well Costar plates with UV transparent flat bottom (Corning Inc., Corning, NY)
Safire Tecan microplate reader (Tecan Group, Männedorf, Switzerland)

Methods:

Nevirapine Extraction

The Nevirapine used throughout these experiments came from two different sources, which were either the pure compound or purified from commercial Viramune tablets. Since Nevirapine pills contain other compounds the Nevirapine had to be extracted and crystallized. The Nevirapine pill was crushed using a mortar and pestle and then weighed in a 50 ml centrifuge tube. Dilute acetic acid (50 mL, pH 2.4) was added to partially dissolve the pill. The sample was incubated 20 minutes, and then centrifuged for 20 min at 3,000 rpm. The supernatant was collected and placed into a second 50 ml centrifuge tube. Acetic acid was added

again to the first tube, incubated at room temperature for 20 minutes and then centrifuged for 20 min at 3,000 rpm as well. The second supernatant was transfer to another 50 mL tube. Both tubes containing the acetic acid extractions were frozen at -80º C and lyophilized overnight. After the sample had completely dried, the Nevirapine was collected and weighed. In order to assess the purity, the compound was characterized by ^{1}H-NMR, IR, and UV-Vis spectra.

Binding to Different Albumin Proteins

Stock solutions of OVA, HSA, and BSA at 50 mg protein/mL in PBS, and Nevirapine at 17 mg/mL in HPLC grade methanol were prepared for this experiment. 1 mL of OVA-NEV, HSA-NEV, and BSA-NEV solutions were prepared by mixing 10 µL of Nevirapine solution (stock solution in MeOH) and 990 µL of 50 mg/mL protein solutions. Nevirapine control solution was prepared by mixing 10 uL of Nevirapine stock solution in methanol and 990 uL of PBS. Additional a blank solution of 1% MeOH/99% PBS solution was prepared as control.

The UV absorbance of stock solutions of Nevirapine, OVA, BSA, and HSA were measured as well as mixtures of Nevirapine with each of the albumin proteins (Nev-OVA, Nev-BSA, and Nev-HSA). 250 µl of each solution was added to labeled 3k centrifugal ultrafiltration tubes. These samples were then centrifuged for 20 min at 3,000 rpm. The volume of the filtrate was measured and all the tubes were adjusted to the same final volume. Then 150 µl of each of the filtrates were transferred to a 96-well plate and the absorbance was measured at 315 nm.

After the absorbance had been taken, 250 µl of 1% MeOH/99% PBS solution was added to each of the tube, and then the samples were centrifuged through 3 k centrifugal filters at 3,000 rpm for 20 minutes. The volume was measured, and then adjusted in all the tubes and then 150 µl was transferred to a 96 well plate. The absorbance of each sample was taken again at 315 nm.

The protein/Nevirapine solution retained in the filter was also processed for measurement of Nevirapine absorption at 315 nm.

Effect of tRNA-HSA polyplex formation on Nev-HSA binding

Prior to beginning this experiment, fresh tRNA solutions were made in PBS at concentrations of 1 mg/mL, 5 mg/mL and 10 mg/mL. A sample of tRNA solution (750 µL) was added to a tube containing 250 µL of a HSA-Nevirapine sample. A control sample was prepared by adding PBS to another tube containing HSA-Nevirapine. These tubes were incubated at room temperature for 20 minutes and then placed in a centrifuge for 5 minutes at 10,000 rpm. 250 µL of each solution was transferred to labeled 3k centrifugal filters and spun for 20 minutes at 3,000 rpm. The filtrate was transferred and measured. Each filtrate was placed in a new Eppendorf tube and labeled "wash 1". 250 µL of 1% MeOH in PBS was transferred to the 3k centrifugal tubes which were spun again for 20 minutes at 3,000 rpm again. The same process was repeated to transfer the filtrate and was labeled "wash 2" for each sample. The volumes of all the tubes were adjusted to be the same volume and then 150 µL of the filtrate was placed in a 96-well plate. The absorbance was then taken at 315 nm to measure Nevirapine concentration.

Loading of Nevirapine inside Glucan Particles

Three methods were evaluated for trapping of Nevirapine within glucan particles. The first method required sequential loading of Nevirapine, protein and tRNA. The second approach consisted of co-loading a Nevirapine/protein mixture followed by trapping with tRNA. Finally, protein/tRNA cores were prepared in glucan particles and evaluated for Nevirapine binding.

- Method A. Sequential loading of core components:

Prior to loading inside glucan particles, solutions of BSA and OVA were prepared in water with concentrations of 50 mg/mL. A 0.1M solution of acetic acid was prepared and the pH was adjusted to 2. This solution was used to prepare a 10 mg/mL Nevirapine solution. Empty glucan particles (5 mg) were used and 25 µL of the Nevirapine solution was added. Control samples were also set up using 25 µL of acetic acid and no Nevirapine. These samples were mixed and incubated for two hours at room temperature. After incubation, the samples were lyophilized over night. An additional 25 µL of acetic acid was then added to each sample, and then incubated for 1-2 hours (acetic acid push) and then the samples were again lyophilized overnight.

OVA or BSA (25 µL) was added to the samples. The serum albumins were allowed to penetrate the glucan particles for two hours on ice and then the samples were lyophilized. The samples were treated with 25 µL of water (water push), incubated for 2 hours at 4 C and lyophilized.

After this step the indicated amounts of tRNA were added to the tubes and then the particles were collected through centrifugation. The supernatants were also collected in labeled Ep-tubes and frozen for later analysis. The particles in the pellet were then re-suspended in 70% ethanol, sonicated then incubated for thirty minutes. The particles were washed three times with saline and then finally re-suspended in 1 mL of saline. The samples were sonicated, counted, and then adjusted to 1x10e8 particles/mL and stored at -20º C.

- Method B. Co-loading of Nevirapine/protein mixture and subsequent trapping with tRNA

A solution of HSA (495 μL of 50 mg/mL in PBS) and Nevirapine (5 μL of 17 mg/mL in methanol) was prepared and added to a sample of GP (5 μL of HSA/Nev mixture/mg particle). The mixture was thoroughly mixed and incubated for two hours at hours at 4 °C. tRNA (250 μL of 10 mg/mL) was added and the particles allowed to swell for 10 minutes, sonicated and then incubated at room temperature for an additional 30 min. The particles were collected by centrifugation, and the supernatants collected in labeled Eppendorf tubes and processed for measurement of unbound Nevirapine. Particle loading was carried out with sterile solvents and in sterile conditions to eliminate the sterilization step with 70% ethanol. Samples were finally washed three times with 0.9% saline, particle number counted with a hematocytometer, and particle dilutions were prepared with a concentration of $1x10^8$ particles/mL and frozen

- Method C. Binding of Nevirapine to GP-protein/tRNA cores

In this method protein/tRNA cores were first synthesized inside glucan particles and used for Nevirapine binding. A solution of HSA, BSA or OVA (50 mg/mL) was mixed with the particles (5 μL/ mg particles) and incubated for 2 hours at 4°C. The sample was lyophilized and 5 μL of water/mg particles was added (water push step). The sample was incubated for an hour and lyophilized. A solution of tRNA (final protein: tRNA ratio of 15) was added to the particles, the sample was sonicated and incubated for 30 minutes. The GP-protein/tRNA cores were washed three times in 0.9% saline, counted and diluted to $1x10^8$ particles/mL and stored frozen. An assay was set up to evaluate Nevirapine binding by titrating Nevirapine concentration from 0 to 2.5 μg/mL. Samples were set up containing 10 μL of $1x10^8$ particles/mL ($1x10^6$ particles), the Nevirapine solution in methanol and PBS to bring the final volume to 100 μL. The Nevirapine was prepared in methanol and the final concentration of methanol in the samples containing

particles was 1%. The samples were incubated at room temperature for 2 hours. Then the samples were centrifuged, and the supernatants carefully transferred to another tube and processed to measure unbound Nevirapine by UV absorption at 315 nm. UV assay for quantitative analysis of Nevirapine

In all experiments, Nevirapine was quantified by measuring absorption at 315 nm of the unbound Nevirapine collected after separation from the particles by centrifugation. The Nevirapine removed from washing the particles was also quantified. The total amount of Nevirapine from the first supernatant and washes was used to indirectly estimate the percentage of Nevirapine bound within the particles. All samples measured by UV assay were processed by addition of spectrophotometric grade methanol to adjust the concentration of methanol to 90% v/v. This allowed for precipitation of tRNA and proteins that would interfere with UV absorption measurements. The samples were centrifuged, and the supernatant transferred to 96-well plates to measure the absorbance. In all experiments, control samples of PBS, tRNA, and proteins were also treated with methanol to assure that the interferents were being removed prior to the UV measurements.

UV Assay for Quantitative Analysis of Nevirapine

In all experiments, Nevirapine was quantified by measuring absorption at 315 nm of the unbound Nevirapine collected after separation from the particles by centrifugation. The Nevirapine removed from washing the particles was also quantified. The total amount of Nevirapine from the first supernatant and washes was used to indirectly estimate the percentage of Nevirapine loaded into the particles. All samples measured by UV assay were processed by addition of spectrophotometric grade methanol to adjust the concentration of methanol to 90% v/v. This allowed for precipitation of tRNA and proteins that would interfere with UV

absorption measurements. The samples were centrifuged, and the supernatant transferred to 96-well plates to measure the absorbance. In all experiments, control samples of PBS, tRNA, and proteins were also treated with methanol to assure that the interferents were being removed prior to the UV measurements.

Evaluation of Nevirapine Release from GP-Nevirapine Formulations

In order to determine whether Nevirapine was being released from the protein/tRNA cores two primary methods were used. The first method used pepsin treatment to determine the amount of Nevirapine released. 100 μL of each sample being tested were transferred to labeled Ep-tubes. The samples were centrifuged and 90 μL of the supernatant were removed. 90 μL of pepsin was then added to the samples, and then the samples were sonicated and incubated overnight at room temperature. After the particles had been incubated, they were sonicated again, and 90 μL of the supernatant was transferred to another tube which was then centrifuged. 75 μL of the supernatants were transferred into a 96-well plate and the absorbance was measured at 315 nm. The samples were also incubated in methanol, HCl, high salt concentrations and EDTA.

The second method for evaluating the Nevirapine release involved feeding the samples to 3T3-D1 cells. This cell line is capable of phagocytosing glucan particles via the dectin-1 glucan receptor.10 μL of the samples of interest were added to 90 μL of saline. Samples containing empty particles as well as samples of pure saline were used as controls as well. Each sample was then mixed with 250 μL of DMEM and transferred to wells containing the 3T3-D1 cells. The cells were incubated overnight, and then the medium was collected in labeled Ep-tubes. The remaining cells were then treated with trypsin and fixed with formalin.

Evaluation of GP-Nevirapine Toxicity to 3T3-D1 Cells

In order to evaluate Nevirapine toxicity, the release of the drug from the protein/tRNA cores in glucan particles was assessed. Cores that contained various formulations of Nevirapine, proteins and tRNA were fed to 3T3-D1 cells. 10 μL of the samples of interest were added to 90 μL of saline. These were then combined with 250 μL of DMEM and transferred to wells containing the 3T3-D1 cells. The cells were incubated overnight and then the medium was collected, transferred to a new plate and then frozen. The remaining cells were then washed with PBS and fixed with formalin.

RESULTS

Nevirapine Extraction

Nevirapine was obtained from the commercial drug formulation Viramune by an acetic acid extraction. Nevirapine has high solubility in aqueous solutions at low pH, while most of the excipients are insoluble in aqueous solvents. The insoluble excipients include microcrystalline cellulose, lactose croscarmellose sodium, povidone, colloidal anhydrous silica, purified talc, and magnesium stearate which make up the pill. Analysis of the extracted compound by NMR (**Figure-8**), IR, and UV-Vis demonstrated that the compound obtained was relatively pure, and suitable for use in the glucan particle encapsulation assays without further purification. Nevirapine was extracted from the commercial pills with a 99.6% yield.

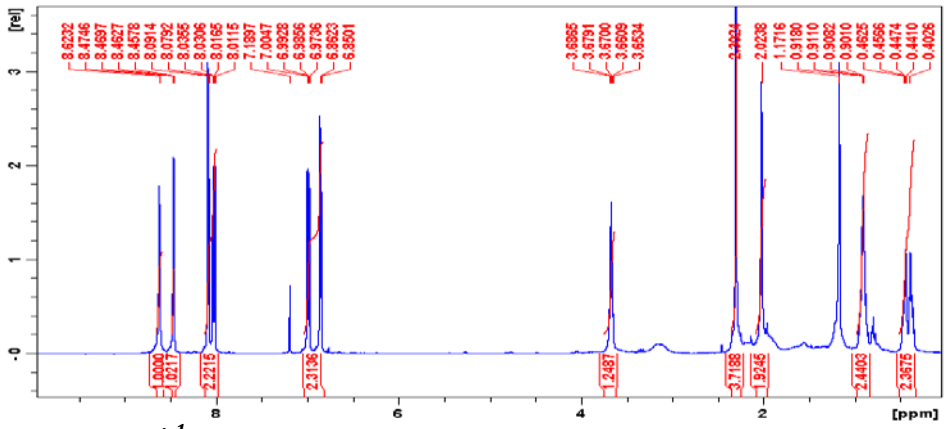

Figure 8: ^{1}H -NMR of Nevirapine extracted from Viramune pills

The ^{1}H-NMR results (**Figure 8**) showed characteristic bands of Nevirapine which matched up with the results obtained from testing a known sample of Nevirapine. **Figure 9** also shows the UV/Vis spectra of Nevirapine in methanol. A maximum absorption peak at 280 nm was measured. Based on the peaks obtained from the NMR it is possible to identify which protons from the structure correspond with each chemical shift. Around 0.45 and 0.95 the shifts are caused by the CH groups on the cyclopropane. In the picture of Nevirapine (**Figure 10**) these

hydrogens, which are mirroring CH2 groups, are identified as 31, 32, 33, and 34. The next visible shifts occur at the methyl group around 2.1 and correspond with the hydrogens 27, 28, and 29. The final shift of interest from the NMR is at 3.2, and this corresponds with the final part of the cyclopropane group and is identified as H_30.

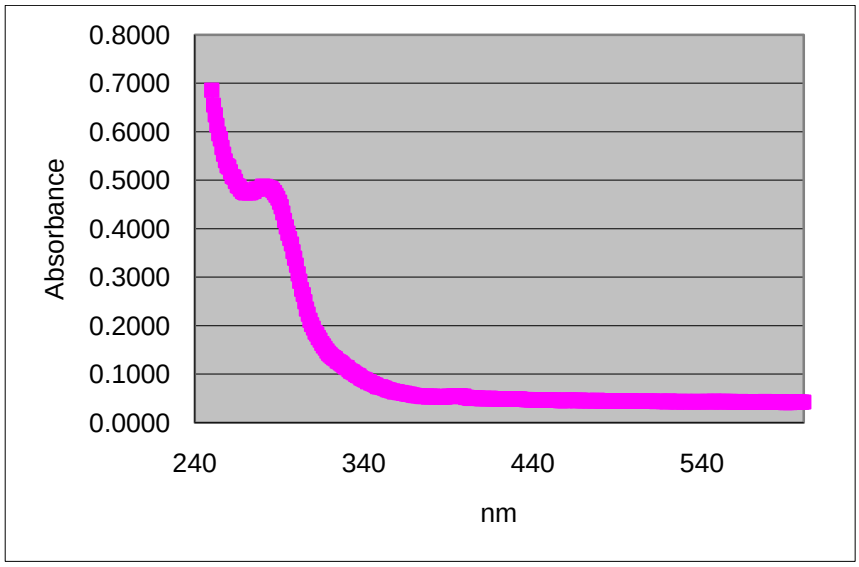

Figure 9: UV-Vis spectra of Nevirapine in methanol

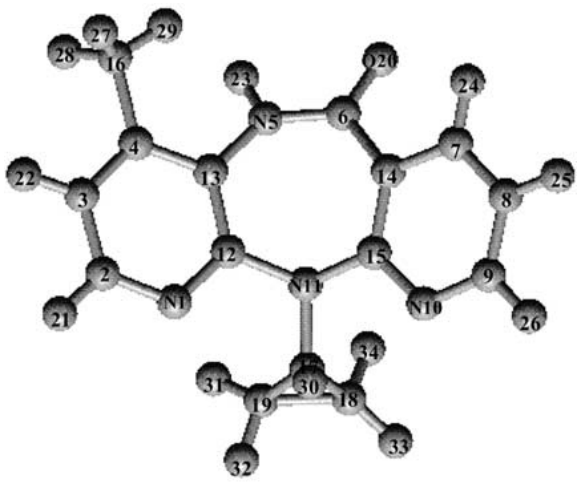

***Figure 10: Nevirapine Structure With Labeled Hydrogens* (Hannongbua et al., 2001)**

Nevirapine Binding

It is known that Nevirapine binds effectively to human serum albumin (HSA) (Bocedi et al., 2004). Encapsulation of proteins (OVA and BSA) within GPs has been achieved by formation of nanoparticulate cores assembled by electrostatic interactions between protein and tRNA (Soto and Ostroff, 2008). Based on the developed protocols for protein encapsulation within GP and the ability of Nevirapine to bind HSA, it was decided to develop methods for Nevirapine encapsulation as part of a protein/tRNA core. The efficiency of Nevirapine binding to different albumin proteins and the effect of protein precipitation with tRNA on Nevirapine retention within the protein/nev complex was evaluated to determine optimal conditions (protein, nevirapine and tRNA concentrations) for GP loading.

Mixtures of the different proteins with Nevirapine were filtered using 3 kDa centrifugal filters to separate unbound Nevirapine from the protein/Nevirapine complex that is retained by the filter due to its high molecular weight. The absorbance of the processed filtrate and retentate was measured at 315 nm to quantify Nevirapine. A wavelength of 315 nm was chosen for this assay as the Nevirapine presents a high absorption and protein or tRNA interference is minimal. Background absorption from the other core components is higher at 254 nm or at the maximum absorption wavelength of Nevirapine (280 nm). Additionally, absorption at 315 nm has been used in HPLC assays that are expected to be implemented to quantify Nevirapine bound to GP samples.

The results shown in **Figure 11** indicate that human serum albumin was most effective in binding the Nevirapine at 83%. Bovine serum albumin and ovalbumin also bound Nevirapine with 54% and 37% efficiency and were used for the preparation of GP-Nevirapine formulations.

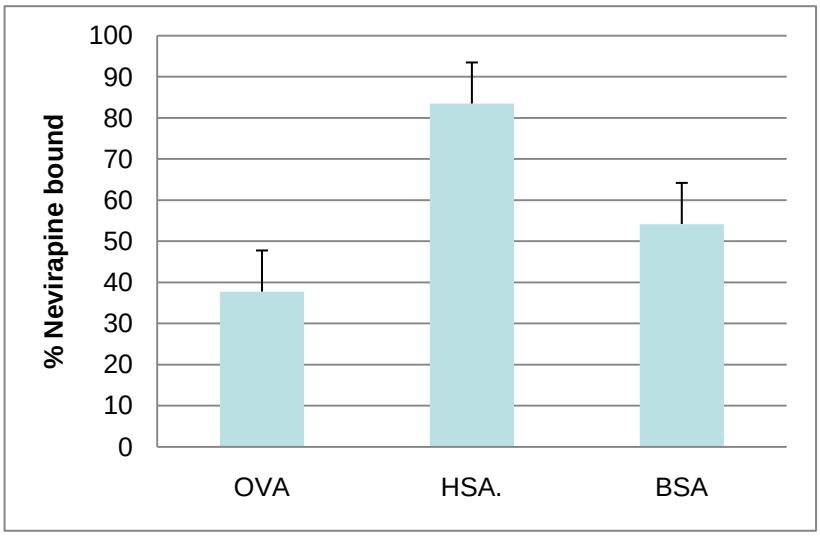

Figure 11: Nevirapine Binding to Different Albumin Proteins.

The second part of this experiment to optimize Nevirapine loading was the evaluation of tRNA interaction with the protein/Nevirapine complex. Specifically, tRNA was titrated to determine the optimal amount of tRNA required to precipitate the protein and retain the drug (Nevirapine) as part of the complex.

The results (**Figure 12**) show that at low concentrations of tRNA (1 mg/mL) the amount of Nevirapine retained is greater than at the higher concentrations of tRNA. At only 1 mg/mL tRNA the percent Nevirapine bound is almost 80% but when the tRNA concentration is increased to 5 mg/mL or 10 mg/mL, the percent bound is only around 40%, nearly half the amount as the lower concentration. tRNA at lower concentrations do not form a precipitate with the proteins. Therefore, the lowest concentration of tRNA (1 mg/mL) capable to precipitate the protein is essential and will be extremely important once we load the drug into the glucan particles.

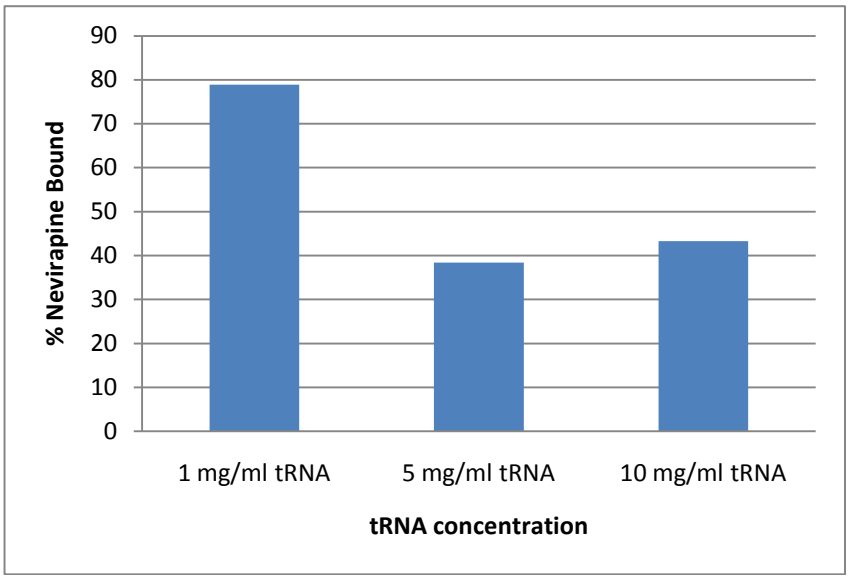

Figure 12: Effect of tRNA Concentration on Nevirapine Bound to the Nevirapine/HSA/tRNA Nanoparticle Complex.

Nevirapine Loading into Glucan Particles

As indicated in the previous section (Materials and Methods), three methods were evaluated for encapsulation of Nevirapine within glucan particles. Essential to keeping the drug and the protein inside the glucan particle was the protein's interaction with tRNA. Since tRNA will precipitate the protein, the Nevirapine will remain bound as long as the tRNA concentration is kept low, around 1mg/mL, as discovered in the previous experiment.

In the first instance of the loading (method A), a 10 mg/mL sample of Nevirapine in acetic acid was used. A half of the hydrodynamic volume (10 µL/mg GPs) of the Nevirapine solution was added to the glucan particles and allowed to incubate for two hours before it was frozen and lyophilized. Nevirapine was added twice to maximize drug loading before addition of the protein and final precipitation with tRNA.

The amount of Nevirapine bound in the cores was indirectly determined by measuring the amount of unbound Nevirapine collected in the supernatant after particle centrifugation and

subsequent particle washings. Sequential loading of the core components (Method A) was used to prepare formulations of Nevirapine with OVA and BSA.

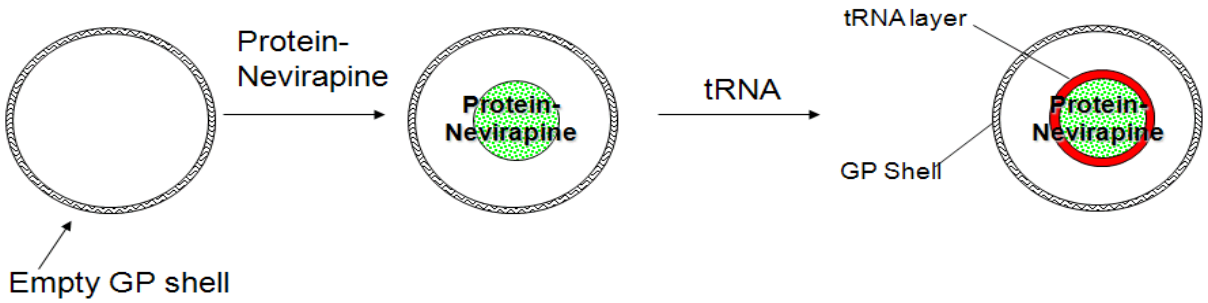

Figure 13: Method B of Loading Protein/tRNA/Nevirapine Cores into Glucan Particles

In the co-loading method (method B) solutions of HSA/Nevirapine were loaded into glucan particles followed by tRNA precipitation of the HSA/Nevirapine complex (**Figure 13**). The amount of Nevirapine bound was also quantified by measurement of the unbound Nevirapine in the supernatants. **Figure 14** shows the results as percentage of Nevirapine trapped inside glucan particles for the best formulations obtained with each of the three albumin proteins. The results of the loading experiments were pretty conclusive. It was determined that the glucan particle core that best trapped the Nevirapine was built using a Nevirapine-HSA-tRNA core. This core yielded a loading efficiency of 62%. The BSA-tRNA core trapped the Nevirapine at 41% and the OVA-tRNA core yielded 29% efficiency. The results of this experiment are expected. The HSA was the best for trapping the Nevirapine and this result was not surprising due to the fact that the HSA had the best binding efficiency with drug.

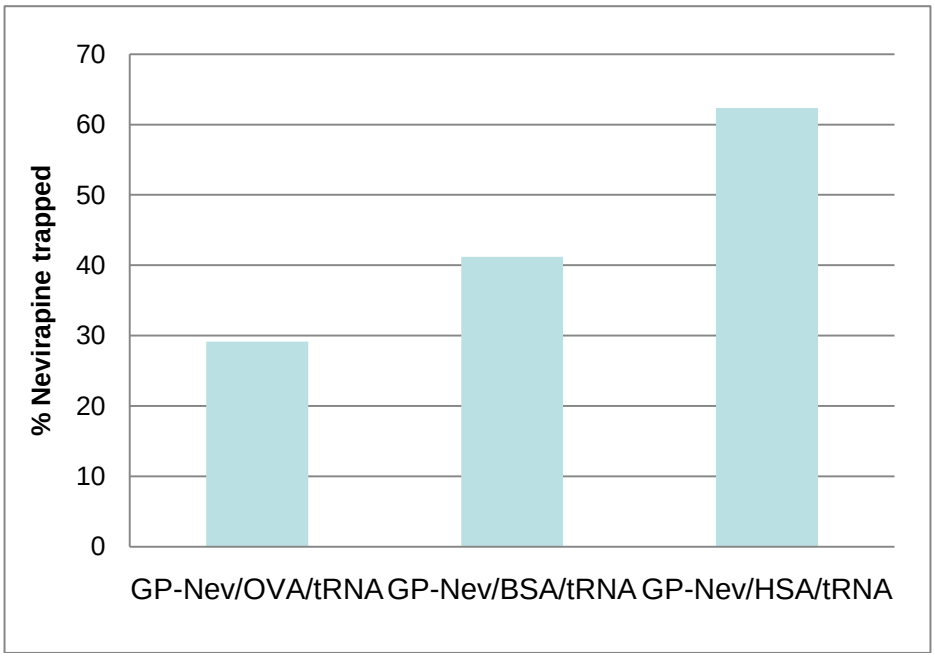

Figure 14: Nevirapine Loading in Glucan Particles

Finally, Nevirapine binding was evaluated using GP-protein/tRNA formulations (Method C) (**Figure 15**). Nevirapine binding to these protein core formulations was evaluated by titrating the amount of Nevirapine incubated with a constant amount of particles ($1x10^6$ particles). The results shown in **Figure 16** demonstrate that all particles containing a protein core efficiently bound Nevirapine. The negative controls (empty glucan particles or particles containing a tRNA/PEI core) show less Nevirapine binding as most the compound was removed after washing the samples.

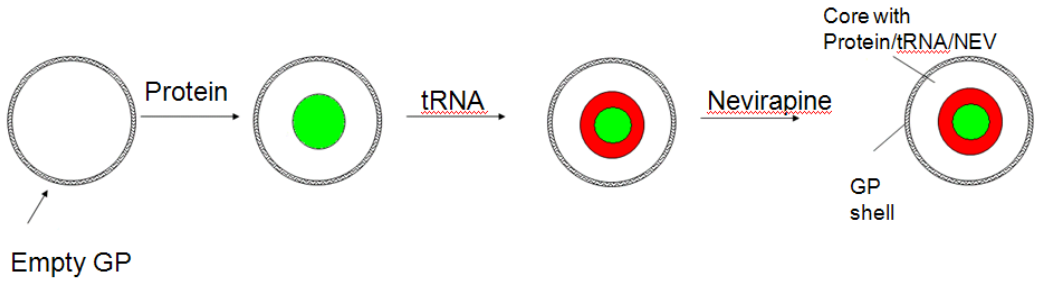

Figure 15: Method C of Loading Protein/tRNA/Nevirapine Cores into Glucan Particles

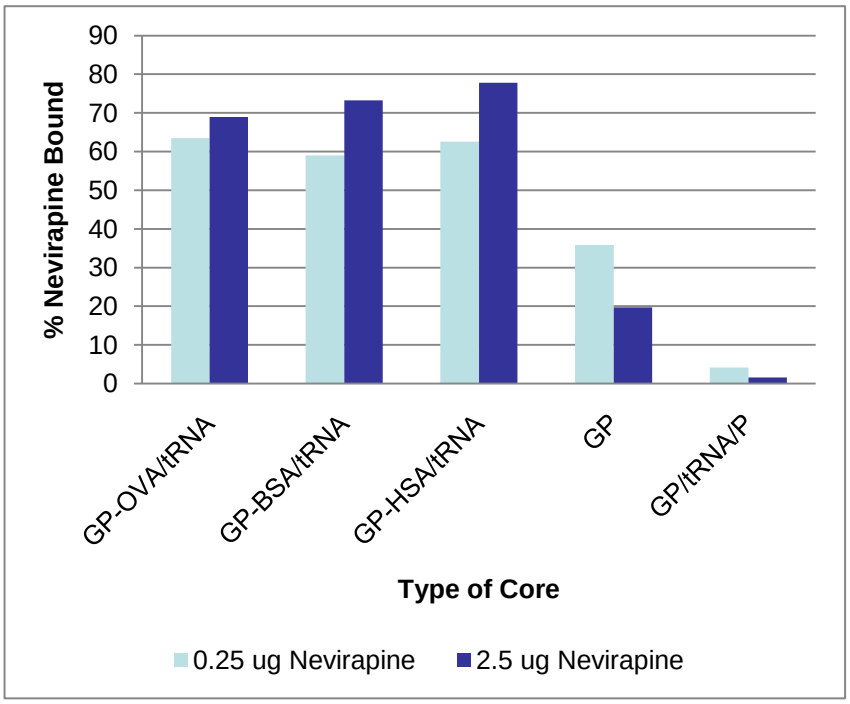

Figure 16: Nevirapine Binding to GP-Protein/tRNA Formulation.

DISCUSSION

Through our project we developed methods to successfully trap Nevirapine inside glucan particles. Glucan particles have been efficiently used to encapsulate macromolecules like DNA and siRNA by formation of nanoparticulate materials assembled by electrostatic interactions between the anionic nucleic acids and cationic trapping polymers like polyethylenimine (PEI). There is a growing interest to extend the applicability of the glucan particle delivery technology to encapsulate small drug molecules. Our project was focused on the encapsulation of the HIV drug Nevirapine for the potential use of GP-Nevirapine formulations as a topical HIV microbicide. Glucan particles have been used to trap protein molecules such as Ovalbumin and Bovine Serum Albumin (BSA) by formation of protein/tRNA nanoparticulate complex. It is also known that Nevirapine binds efficiently to albumin proteins, specifically to human serum albumin. Taking into consideration the binding ability of Nevirapine to HSA and the possibility of forming protein/tRNA cores within GP it was determined that the best approach to attempt trapping of Nevirapine is by formation of Nevirapine/albulimin-protein/tRNA cores.

To assess the best conditions for formation of these cores, the binding of Nevirapine to different albumin proteins was measured by UV. As shown in **Figure 10**, HSA efficiently binds the drug confirming previous studies that have reported binding of Nevirapine to HSA with 70% higher efficiency. It was found that OVA and BSA also bind Nevirapine and subsequently these proteins were also evaluated for Nevirapine trapping inside GPs. A critical step in the formation of Nev/protein/tRNA cores is the precipitation of the Nevirapine/protein complex upon addition of tRNA without significant loss of Nevirapine. Typically GP-protein/tRNA cores are prepared using a high ratio of protein: tRNA (1:10). However this high ratio of tRNA was found to

displace Nevirapine from the complex. The tRNA titration results (**Figure 11**) showed that a protein: tRNA ratio of 1 allows for effective precipitation of the protein/Nevirapine complex without significant loss of Nevirapine. A lower tRNA: protein ratio does not effectively forms a tRNA/protein precipitate.

Loading of Nevirapine within GP and trapping into a tRNA/protein core was investigated by three methods. The first method was the sequential loading of the drug components. Efficient adsorption of payloads into glucan particles was achieved by soaking dry particles in half of the hydrodynamic volume of the particles. The wet sample is then lyophilized before addition of the next component of the core formulation. This method guarantees maximum adsorption of each component. The final compound added to this formulation is the trapping polymer (tRNA) that forms the stable nanoparticulate complex trapped within the particles. This method was applied to form Nevirapine/protein/tRNA cores with OVA and BSA. The results of **Figure 12** show that the best formulations contain less than 50% of the input Nevirapine.

In the second method, the drug and protein were co-loaded into the glucan particles and then tRNA was added to precipitate the protein/Nevirapine complex. In this method the lyophilization steps were not carried out as it is possible that the cycles of drying and re-suspending the samples had a negative effect on Nevirapine solubility and its interaction with the re-dissolved protein resulting in poor Nevirapine retention inside the particles. This method allows for efficient trapping of the HSA/Nevirapine as shown in **Figure 12**.

The final method evaluated to trap Nevirapine was the use of glucan particles containing a pre-formed protein/tRNA core. If the binding sites of the protein are still available after complex formation with tRNA, it should be possible to absorb the drug in a similar fashion to the LbL assembly of plasmid DNA or siRNA onto glucan encapsulated cationic cores. To guarantee

that Nevirapine would bind, the protein/tRNA cores were prepared using the minimum tRNA: protein ratio that allows for protein core formation. The results from **Figure 13** show that the cores were effective in binding Nevirapine with more than 50% binding at different drug concentrations.

Three methods to successfully trap the Nevirapine have been developed and these methods need to be optimized. For Methods A and B, the concentration of Nevirapine was constant the entire time. The last loading experiment that built a protein-tRNA core and then allowed the Nevirapine to be absorbed was the only experiment where the concentration of Nevirapine was varied. In order to optimize the loading, the concentration of Nevirapine should be varied. It was discovered that the 2.5 μg of Nevirapine yielded a higher trapping efficiency than the 0.25 μg, but varying the concentrations will allow for the optimal concentration to get the best binding rate.

The amount of Nevirapine bound to the particles was indirectly determined by a UV assay. Characterization of the samples still requires optimization. The best method that will provide a direct measurement of bound Nevirapine is the use of HPLC. Different GP-Nevirapine formulations were processed to (1) facilitate release of the drug from the formulation by incubating the samples with pepsin to degrade the protein, different salt concentrations, pH, and solvents to release the drug from the cores, and (2) treatment of the whole particle sample with DMSO to dissolve the particles and analyze the final mixture by HPLC. Future work will focus on optimization of HPLC protocols to quantify total Nevirapine bound in GPs and Nevirapine release. In **Figure 13**, the only result that was slightly skewed from what was expected came from the cores that contained only glucan particles. Since there was no Nevirapine in the samples there shouldn't have been any Nevirapine found when evaluated by UV spectrum. This could

have been caused by improper washing or inefficiency when washing the samples. This also proves that another method to quantitate the Nevirapine contents of the samples is necessary in order to properly examine the cores.

Different GP-Nevirapine formulations were evaluated for toxicity effect on the murine fibroblast cell line NIH3T3-D1. Particles were fed at ratios of 1, 3.3, 10 and 33 particles/cell. After analyzing the results from this experiment, the Nevirapine formulations had no visible effects or toxicity on the cells.

FUTURE WORK

After performing experiments on Nevirapine extraction, binding, and loading, it is clear that this drug has the potential to be used to create a successful HIV microbicide. The methods developed have allowed for Nevirapine to be trapped inside HSA-tRNA cores with an optimal binding efficiency greater than 70%. In the future, the next step needed to continue progressing forward is a more in depth analysis of the samples by HPLC. The binding efficiency can be assessed in a much more exact manner through the use of HPLC. By creating a concentration gradient using samples containing known amounts of Nevirapine, it will be possible to determine exactly what is binding within the cores. Different concentrations of Nevirapine can also be used to optimize loading. The rate of Nevirapine released from the particles is also a key factor. If the Nevirapine is not properly released from the cores, then the Nevirapine may have trouble being delivered to macrophages and Langerhans cells, which is the ultimate goal of this microbicide. The rate needs to be determined in order to make sure that this is a viable method of preventing HIV infection.

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APPENDIX A

Tube	**HSA-Nevirapine µL**	**Trapping Polymer**	**µL**
1	250	PBS	750
2	250	1 mg/ml tRNA	750
3	250	5 mg/ml tRNA	750
4	250	10 mg/ml tRNA	750

1. Prepare the following mixtures:
   10 uL of Nevirapine in MeOH + 990 uL PBS
   1 mL of HSA-Nev solution by mixing 10 uL of Nevirapine solution (stock solution in MeOH) and 990 uL of protein solution
   1, 5, and 10 mg/mL tRNA in PBS
2. Measure 250 uL of HSA-Nev. Add indicated amount of tRNA or PBS
3. Incubate 20 min at room temperature
4. Centrifuge samples 5 min at 10 rpm
5. Transfer 250 uL of sample to labeled 3 k centrifuge filters
6. Centrifuge samples 20 min at 3000 rpm
7. Carefully transfer filtrate to labeled Ep tubes and measure total volume
8. Add 250 uL of 1%MeOH/99% PBS solvent mixture to each tube.
   Centrifuge samples 20 min at 3000 rpm
9. Carefully transfer filtrate to labeled Ep tubes (wash 1) and measure total volume
10. Repeat steps 6-7 to collect wash#2
11. Adjust volumes of all Ep tubes to same final volume
12. Transfer 100 uL to 96-well plate
13. Measure Abs @ 315 and calculate % of unbound Nevirapine

tube	mg GP	µL 0.1 M aa	protein	µL protein	mg/ml tRNA	µL tRNA
1	5	0	50 mg/ml OVA	25	1.5	500
2	5	0	50 mg/ml BSA	25	1.5	500
3	5	25	50 mg/ml OVA	25	1.5	500
4	5	25	50 mg/ml BSA	25	1.5	500

1. Use 5 mg YGP in Ep tubes
2. Prepare 0.1 M acetic acid. Check pH and adjust to pH 2
3. Prepare a 10 mg/mL solution of Nevirapine in acetic acid
4. Add 25 uL of Nevirapine solution to GP. Add acetic acid to control samples. Mix w/blunt pipette tip until wet
5. Incubate 2 hours at room temperature
6. Freeze, lyophilize
7. Add 25 uL 0.1 M acetic acid. Incubate 1-2 hours
8. Freeze, lyophilize
9. Prepare 50 mg/mL OVA in water, 50 mg/mL BSA in water
10. Add 25 uL of OVA or BSA solution to GP-Nevirapine (5 uL 50 mg/mL OVA/BSA /mg

GPs)

11. Mix w/blunt pipette tip until wet and allow OVA or BSA to penetrate GP for 2 hours on ice
12. Freeze/lyophilize
13. Carry out water wash by adding 25 ul saline to tubes. Incubate 2 hrs 4C.
14 Add indicated volume of tRNA at indicated concentration in 0.9% saline at 50C
15. Allow to swell for 10 minutes at 50C, sonicate to single particles, allow to coat for 20 minutes
16. Collect particles by centrifugation. Collect supers into labeled Ep-tubes. Freeze for Nevirapine analysis
17. Wash 3X in 0.9% saline - resuspend by sonication - Collect supers into labeled Ep-tubes. Freeze for Nevirapine analysis
18. Resuspend in 70% ethanol - sonicate, incubate at least 30 min
19. Wash 3X in 0.9% saline - resuspend by sonication Resuspend tubes in 1 ml saline
20. Sonicate, count, adjust aliquot to 1x10^8 particles/ml.
21. Store tubes at -20C

Tube	**mg GP**	**Loading solution**	**µL**	**mg/mL tRNA**	**µL tRNA**
1	5	HSA-NEV	25	1	250
2	5	HSA	25	1	250
3	5	HSA-NEV	25	1	250
4	5	HSA	25	1	250
5	0	HSA-NEV	25	1	250
6	0	HSA	25	1	250

1. Prepare a 0, 0.001, 0.01, and 0.1 mg/mL Nevirapine solution in PBS
2. Measure UV absorbance @ 315 nm of Nevirapine solutions before setting up experiment
3. Mix indicated amounts of PBS, Nevirapine and particles
4. Incubate samples for 1 hour
5. Centrifuge samples. Carefully transfer 90 uL of SN to 96-well plate
6. Add 90 uL of PBS to each tube. Sonicate sample, vortex and transfer to 96-well plate
7. Measure UV absorbance @ 315 nm

1. Prepare the following mixtures:
   10 uL of Nevirapine in MeOH + 990 uL PBS
   1 mL of OVA-Nev and BSA-Nev solutions by mixing 10 uL of Nevirapine solution (stock solution in MeOH) and 990 uL of protein solution
2. Measure UV absorbance @ 315 of stock solutions of mixtures of OVA/Nev, HSA/Nev, and BSA/Nev, and Nev solution in 1% MeOH
3. Transfer 250 uL of indicated solutions to labeled 3 k centrifugal filters
4. Centrifuge samples 20 min at 3000 rpm
5. Carefully transfer filtrate to labeled Ep tubes and measure total volume
6. Add 250 uL of 1%MeOH/99% PBS solvent mixture to each tube.
   Centrifuge samples 20 min at 3000 rpm
7. Carefully transfer filtrate to labeled Ep tubes (wash 1) and measure total volume

8. Repeat steps 6-7 to collect wash#2
9. Adjust volumes of all Ep tubes to same final volume
10. Transfer 100 uL to 96-well plate
11. Measure Abs @ 315 and calculate % of unbound Nevirapine