Multifunctional Therapeutics using Engineered Plant Virus Nanoparticles

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Nanotechnology holds great promise for cancer therapy, but difficult chemistry problems have impeded progress in efficacy, elimination of side effects, and commercial production. These problems are not a factor with the engineered plant virus nanoparticle that will be used in the proposed research. Through millions of years of evolution, this unique virus has solved production, uniformity, biologic, and pharmacokinetic problems. This plant virus, is in the human food chain, is nontoxic, and is of low immunogenicity. It can load medical imaging agents and/or therapeutic drugs into its "cargo chamber" by a simple process. The virus can not only be engineered to identify and enter cancer cells but can target and release drug payloads inside cell structures, such as the nucleus where most chemotherapy drugs operate, bypassing most cancer cell defenses. In this project, we will implement a new approach to increase production of these plant virus nanoparticles. We will also demonstrate effective delivery of imaging agents and chemotherapy drugs to drug resistant cancer cells and to tumors in an animal model, in preparation for a possible Phase II research project leading to further development of the best drug formulation and an IND application for clinical trials.
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A. Identification and Significance of the Problem and Opportunity

Although considerable progress has been made in the application of nanotechnology to cancer therapy, there are central issues impeding or preventing the currently diverse paradigmatic set of nanoscale approaches from actualizing certain goals. **One example of an un-actualized goal is the ability to provide highly efficacious targeted drug delivery to tumors without adverse effects.** A general theory as to why this goal has not yet been actualized is that some of the inherent challenges associated with nanoparticles, for example: efficient manufacturing of nanoparticles; uniform loading; and timely cargo delivery; involve very difficult chemistry problems, some of which do not at this time have suitable solutions.

Our nanoparticle inherently overcomes these problems because it is a biologic, specifically the product of natural replication of a unique plant virus in a well-controlled host plant system involving minimal human intervention. This unusual virus brings with it some very interesting properties that turn out to be highly desired for a nanoscale drug delivery system. Each nanoparticle is identical and can be filled with equal amounts of drug cargo using a simple process. Moreover, the best chemistry-based technologies have been as yet unable to achieve the equivalent to what this plant virus has achieved through millions of years of evolution, such as the ability with the proper targeting to: form endosomes and enter cancer cells; sense the intracellular environment and initiate a process leading to the release of the contents of its “cargo chamber”; and delay this release long enough to enable the secondary targeting of intracellular structures, such as the nucleus. The natural first clinical application of this virus is oncology, where antitumor drugs can be delivered to the nuclei of tumor cells, dramatically increasing efficacy and tolerability while bypassing the main mechanism of resistance, cytosolic efflux pumps. There are numerous additional clinical applications involving multiple therapeutic areas. Potential nanoparticle cargo molecules may include: drugs (including those otherwise ineffective due to pharmacokinetic issues); dyes; peptides; nucleotides (such as siRNA or other gene silencing technologies); and possibly others.

Some more specific reasons as to why the anticancer therapy goal of various nanotechnologies has not yet been actualized include:

1) **Inadequate technologies enabling effective therapeutic cargo loading in nanoparticles**
   a. Nanoparticle polydispersity
   b. Poor nanoparticle reproducibility
   c. Fundamental manufacturing issues

2) **Poor biological function**
   a. Inability to deliver cargo inside a cell
   b. Premature release of cargo
   c. Non-release of cargo

3) **Lack of oncology-specific advantages**
   a. Inability to differentiate healthy cells from cancer cells
   b. Inability to overcome multidrug resistance (MDR)
   c. Inability to achieve tumor penetration, and others.

![Figure 1](image)

**Figure 1.** Preparation and use of PVN cell targeting agents. A. Preparation of loaded PVNs. B. *In vitro* cell targeting experiments.
We believe that the engineered plant virus we are using can adequately address all of these reasons for the serious difficulties presently encountered using nanoparticle-based therapies. In our proposal, this plant virus nanoparticle (PVN) based platform is presented and employed as a multifunctional nanoparticle, and we will present preliminary data supporting this utility. Our multifunctional nanoparticle platform may serve as a carrier for imaging agents as well as for therapeutics and is capable of cellular and sub-cellular targeting. Figure 1 shows how this platform may serve as a generalized test vehicle to establish the effectiveness of cell targeting strategies for many types of targets and targeting molecules. Fig. 1A shows the steps for preparing a PVN. The loading mechanism is novel and results in a uniform population of identical nanoparticles that are identically loaded. The loaded PVN in Fig. 1 carries a fluorophore inside and has targeting peptides on the outside, demonstrating the simplicity of proof-of-concept experiments. Preliminary data demonstrate a highly controlled release of fluorophores inside the targeted cell and not externally. The triggered release does not occur in the endosome due to the low pH and relatively high calcium ion concentration. As shown in Fig. 1B, release occurs in the cytosol and is triggered by cytoplasmic entry. Fluorescent dyes are quenched while inside the PVN, but they can be detected when they are released. The PVN can also carry an internal payload of other types of imaging agents, chemotherapy agents, and small molecules in general. This includes oligonucleotides, as well, and we have already been approached by some of the largest pharmaceutical companies regarding the delivery of oligonucleotides (siRNAs) for gene silencing applications. This therapy option, however, will not be part of this Phase I study as it requires additional PVN manipulation.

We believe that our company, NanoVector, Inc. (NVI) has in place a world-class technology and product development team for future PVN commercialization. Dr. Bruce Oberhardt, the Principal Investigator, has had an extensive career in: industry; small, venture-backed companies; and consulting. He has led and/or participated in a number of first-of-a-kind technological developments, including: early research on thermal kinetics of cell bound antigen-antibody systems; development of novel diagnostic technologies using liposomes and other particle-based carriers; development of technologies for: automated blood chemistry and cell analyzers; novel diagnostic systems; novel molecular and cell separation systems; drug delivery systems; blood coagulation analyzers; and others. Dr. Paul M. Steed has 10 years of pharma (Novartis) and 6 of biotech experience and most recently led the development of pharmaceutical products for oncology and other therapeutic areas at Serenex, recently acquired by Pfizer. He is an expert in cell biology and pharmacology and has performed extensive animal testing and preclinical development of pharmaceuticals. Dr. J. Chris Luft is a cell biologist with wide-ranging experience, including the development and testing of earlier generation, non-biologic nanotechnology-based initiatives in cancer therapy. Ms. Mei Hu is an expert in purification of macromolecules and biologics with experience in pharmaceutical product development. Dr. Stefan Franzen (NC State University (NCSU), Founder of NVI, a biophysical chemist who performed some of the earliest ground-breaking investigations using gold nanoparticles to target cancer cells to achieve endocytosis and endosomolysis is active in the company; Dr. Steven Lommel (NCSU) Cofounder of NVI, a plant virologist and possibly the world’s expert on the unique plant virus that forms the core of the PVN technology is also active in the company; Dr. David Adams, an oncologist with a background in anticancer drug discovery and development that spans eleven years in the pharmaceutical industry and another eleven years up to the present at Duke University Medical Center is a consultant to this project.

In addition to its own laboratory and office space, including equipment, reagents, and supplies relevant to this project, NVI has a sponsored research agreement with the
laboratories of Professors Franzen and Lommel at NCSU and agreements with NCSU enabling the company to have access to capital equipment such as: a flow cytometry system; confocal microscopes; MALDI-TOF analyzers, and other equipment. NVI also has an agreement with NCSU for the use of glasshouse space to grow and harvest the virus needed for this project. The company has longer term plans to expand virus and PVN production to a GMP facility and has already chosen a site for this operation.

The current manufacturing process for the PVN is based on the plant virus: Red Clover Necrotic Mosaic Virus (RCNMV). RCNMV, an extremely robust soil virus found in the human food chain, infects a variety of host plants, including cherries. At present, the front end of the manufacturing process for the PVN entails producing the virus in host plants, 4–6 week old Nicotiana clevelandii, in a greenhouse under controlled conditions. We have also investigated hydroponic and bioreactor-based approaches. Virions are harvested from plants 7-10 days post inoculation and purified as described in greater detail later in this proposal using a series of centrifugation steps. A typical purification yields 70-200 µg of RCNMV per gram of infected plant tissue. Once purified, this unique plant virus can then be filled with a cargo of small molecules, such as dyes or drugs by means of a reversible process involving exposure to low concentrations of magnesium and calcium ions to open holes in the virion allowing infusion of the cargo. The virus is subsequently closed by raising the divalent ion concentration to levels comparable to those in blood and interstitial fluid. Targeting peptides are then covalently conjugated to the lysine terminal surface groups and/or the cysteine terminal surface groups on the viral capsid, of which there are 360 and 180 copies, respectively.

The PVN is stable, robustly protects its cargo, and may be lyophilized for long term storage. The targeting peptides drive the specificity of the targeted cell type (such as a proliferating cancer cell) via avidity binding, and the PVN subsequently enters the targeted cells via endocytosis. The PVN does not begin reopening the holes and releasing cargo until it escapes from the endosome and encounters the low calcium ion concentration in the cytoplasm. From this point in time, the holes begin to reopen in a process taking about three hours, which allows sufficient time for the targeting of structures within the cell, such as the nucleus, where most cancer chemotherapy drugs function and thereby circumventing most resistance mechanisms to chemotherapeutics.

In summary, PVN technology has the potential to overcome all of the current shortcomings of nanoparticle-based therapeutics while advancing the nanotechnology field further via cellular and sub-cellular targeting. Taking advantage of the natural properties of RCNMV, the PVNs can be: produced copiously in a completely homogeneous manner; easily loaded with a variety of payloads; and deployed to deliver cargo on a sub-cellular basis in a highly controlled manner. Subsequent manipulations in the manufacturing process enable PVN’s to target specifically cells and cellular organelles and thereby to achieve superior therapeutic benefit and therapeutic index.

In this proposal we provide preliminary in vitro data on cell targeting with PVNs containing chemotherapy agents and early data obtained from preliminary animal studies in mice indicating that the protein shell of the PVN itself is non-toxic, appears to be non-immunogenic and shows no accumulation in organs prior to excretion via the renal system. It is our observation that from the point of manufacture and loading of drug cargo into the PVN “cargo chamber” the drug is not released until after the PVN enters a cell and senses the cytosol. It should also be recognized and emphasized that the plant virus employed: a) has no inherent targeting capability unless engineered, as in this project; b) does not infect mammalian cells; and c) is in the human food chain and found in familiar foods. Herein we propose to develop further a PVN delivery platform that can be evaluated in detail as a targeted tumor imaging agent delivery system as well as a new therapeutic strategy for the treatment of cancers.
Using the same targeting PVN for detection and treatment of a tumor ensures tumor-specific drug delivery and therefore maximum therapeutic benefit. The goal is to develop an optimized drug-carrying, targeted PVN-based therapeutic and progress into IND-enabling studies in a potential Phase II project.

An additional objective of this proposal is to make PVNs available to investigators in government laboratories, academia, and industry where the numerous possibilities of cargo and targeting combinations can be easily investigated. We believe that this paradigm, if realized, would advance research efforts on many levels to develop more efficacious nanotechnology-based products.

B. Technical Objectives

Aim 1. Improve the current PVN manufacturing process by incorporating a cross flow (tangential flow) filtration technology for virus purification to show feasibility for a PVN production process scalable to essentially unlimited industrial production volumes. Using this technology, demonstrate high batch to batch consistency of product purity. In Aim 1, we propose to improve production of the PVN by focusing on the current “bottleneck” in the PVN production process: virus purification. The PVN is easily produced in quantity, but improved virus purification, could enable scale-up to high volume, providing sufficient quantities of PVN for preclinical development and clinical trials of as well as ample amounts of PVNs that could be produced for and supplied to other researchers and product developers in government, academia, and industry. We will attempt to answer the question: “Can we develop a scalable virus purification step to enable the PVN production process to become scalable to very large volumes?” The cross flow (tangential flow) filtration method we will use is state-of-the art for macromolecules, including viruses, and enables rapid and efficient GMP-compatible purification.

Aim 2. Identify optimal tumor cell and nuclear targeting peptides for PVNs, and develop imaging PVNs. In Aim 2 we will screen a set of tumor-targeting peptides to determine the optimal PVN targeting array. This will include coupling a nuclear targeting sequence to PVN, since the PVN platform is capable of cytoplasmic and nuclear targeting. Both drug delivery destinations will be studied and used to optimize targeting. The PVN will also be labeled using fluorescent targeting peptides to determine peptide loading on the PVN surface. We will screen peptides that target N-cadherin sites on cancer cells for the initial product from NVI technology, because this approach has a large body of supporting data, especially for melanoma (1). Questions we will attempt to answer are: “What likely imaging modalities could be used in animal pharmacology?” We will utilize the optimal targeting PVN to develop a tumor imaging nanoparticle by loading with fluorescent dyes for histological testing. The approach is similar to that described in Figure 1 but will be extended in Aim 4 to animal proof-of-concept studies by the use of a fluorescence imaging PVN appropriate for animal pharmacology. We will address the additional question: “Could this approach enable imaging in conjunction with therapeutic approaches for future testing in a clinical setting?” For example, it should become feasible to image the results of a tumor targeted with a PVN containing contrast agent cargo and then use the same targeting PVN to deliver a therapeutic drug. We will load the PVN with a cargo of clinically validated magnetic resonance imaging (MRI) contrast agent(s) and perform in vitro testing for signal to noise ratio. Specific targeting of tumors using MRI contrast agents, such as gadolinium complexes, should significantly reduce side-effects associated with these agents, e.g. kidney toxicities due to gadolinium. In a possible Phase II proposal, these PVNs could be validated in vivo.
Aim 3. Develop PVN-based formulations for delivery of therapeutic drugs to cancer cells; test these formulations in vitro against tumor cells and compare efficacy with free drug. In Aim 3, the optimized PVN will carry a chemotherapeutic agent inside and display targeting peptides on the outside. We will test the efficacy and loading capacity of well-established chemotherapeutics on a tumor cell panel using a standard ATP-based cell viability assay. We will address the question: “Which formulations outperform the others, and which one is best?” The PVNs will be evaluated in detail to determine potency against several human tumor lines, including resistant lines, since nuclear targeting should thwart resistance via efflux pumps.

Aim 4. Perform in vivo proof-of-concept animal studies with PVNs produced via Aims 1-3 using both imaging and therapeutic PVNs. Compare efficacy of PVN-delivered drug with free drug. Determine xenograft tumor response while monitoring tolerability by clinical observations. In Aim 4, proof-of-concept small animal studies will be conducted using PVNs from Aim 2 loaded with fluorescent tracer as well as the most efficacious drug PVN formulations from Aim 3. Our question here is: “Can we demonstrate improved efficacy as compared to free drug using PVN-enabled cytoplasmic and nuclear targeting?”

In a possible Phase II project, this in vivo work would be expanded to include multiple efficacy and pharmacokinetic (PK) studies along with other IND-enabling activities and might also include the detailed evaluation of targeted PVN’s loaded with the optimal MRI contrast agent from Aim 2. State-of-the-art in vivo tumor imaging could provide diagnostic data and enable detailed evaluation of efficacy in the clinic.

C. Work Plan
Nanovector PVNs are currently in an early pilot production phase, and the utility of the PVN platform for coupling targeting peptides and for loading therapeutic and/or imaging reagents has already been demonstrated. The targeting of cancer cells has been demonstrated in vitro, and improved PVN cytotoxicity over free drug has been amply demonstrated, as well. Therefore, the overall goal of the proposed project is to optimize several aspects of the technology to generate clinically-enabling processes and therapeutically-optimized molecules. This effort is designed to deliver engineered nanoparticles for the clinical plan, which involves applying a matched set of identically targeting PVNs, one with an imaging reagent and another with an anti-tumor drug. According to this plan, patients will be imaged via intravenous delivery of PVN with contrast agent to evaluate tumor burden and determine if their tumors are targeted by the PVN. If so, the patient will be given the drug-PVN, which will deliver the drug to the tumor cells or tumor cell nuclei.

(*)Task 1. Incorporate a cross flow (tangential flow) filtration technology with hollow fibers to provide a virus purification process that is efficient and scalable to high volumes. Using this technology, demonstrate high batch to batch consistency of product purity. We propose to acquire and implement a tangential flow system appropriate for efficient viral purification: a Spectrum Laboratories SYR2-U20-01N Universal KrosFlo Research II TFF System. Optimization will utilize uninfected plant tissue spiked with purified RCNMV loaded with a fluorescent tracer (ethidium bromide) to enable rapid determination of the optimal hollow fiber filters for the low and high molecular weight cutoffs. Virus will then be purified by this method and purity determined using previously established analytical methods with the existing virus purification methodology as a control. Virions are currently purified as shown in Fig. 2 below with final concentration determined by UV spectroscopy (resulting extinction coefficient of 6.46 cm2/mg at 260 nm). Virion concentration is confirmed using the
Coomassie Plus Protein Assay Reagent. A typical purification yields 70-200 µg of RCNMV per g of infected tissue, averaging 50 mg/batch. PVN’s will then be evaluated for efficiency of coupling targeting peptides and competency to load cargo. Fig. 2 shows

**Current Process (50mg yield)**

(*) Figure 2. Current and Proposed Virus Purification Process (indicating recycle for higher levels of purification) the current virus purification process and the proposed new process that we intend to implement in this Aim of the proposed project. The major reason that the new process is highly scalable to large volume production is that the ultracentrifuge in the current process, which is not scalable, is eliminated. All other units of equipment or their equivalents are highly scalable to commercial production size.

(*) Task 2. Identify optimal tumor cell and nuclear targeting peptides for PVNs. We propose to screen peptides that target N-cadherin for the initial product from NVI technology; because this approach has a large body of supporting data, especially for melanoma (1). We plan to test ADH-1, ADH-10, ADH303 and ADH304 to determine the best N-cadherin targeting peptide for the PVN’s. Targeting of N-cadherin positive cancer cell lines [MX-1 (breast), H1299 (lung), and A375 (melanoma)] vs. N-cadherin negative cancer cell lines [MCF7 (breast), PC3 (prostate), and HT29 (colon)] will be evaluated. Peptides will be obtained from GenScript and/or MWG-Biotech and attached using a covalent coupling protocol developed for this purpose based on the heterobifunctional linker SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate). PVNs at various peptide loading densities will be filled with doxorubicin (DOX) using a divalent ion-controlled infusion protocol developed for this purpose. Cell lines will be purchased from the American Type Culture Collection (ADCC) and grown in controlled temperature incubators in the presence of 6-7% CO₂ at pH 7.4 using the ADCC media. The peptide-coupled PVNs with and without DOX will be compared to untargeted PVNs loaded with DOX, and tested vs. free DOX. ATP assays will be used for cell viability determinations using the Invitrogen, A22066, ATP Determination Kit. PVNs will also be coupled with a nuclear localization signal (NLS) and the effects on potency against human tumor cells that over-express efflux pumps (MDR, pGp) vs. their non-over-expressing counterparts evaluated.
The optimal tumor-targeting PVN will then additionally be coupled with NLS and in vitro potency evaluated as above.

**Task 3. Load targeting PVNs from Task 2 with a fluorescent tracer for animal studies.** This tracer can determine the tissue, cellular and sub-cellular location of the PVNs in tumor-bearing mice. Tracers will be loaded into PVNs and tested for best visualization of cellular and nuclear localization in vitro using targeting described in Task 2 and fluorescent microscopy; tracers to be tested: fluorescein, rhodamine, Cy3, Cy5, and indocyanine green. The tracer selected will be used in a PVN for histological examination of tumors and normal tissues in Task 5. In a possible Phase II study, we intend to perform whole animal imaging studies using the imaging PVN from Task 7.

**Task 4. Develop PVN-based formulations for delivery of therapeutic drugs to cancer cells; test these formulations in vitro against tumor cells and compare efficacy with free drug.** It is efficient to use well-established drugs as PVN cargo to streamline the effort in early clinical evaluation. Preliminary data have indicated that doxorubicin loads with high efficiency into PVNs (1,000 molecules per PVN). Using the targeted PVNs from Aim 2, we propose to test doxorubicin (DOX), a topoisomerase 2 (TOPO2) inhibitor in xenograft efficacy proof of concept work. We will also test in vitro commercially available, clinically validated and commonly prescribed antitumor agents: paclitaxel, cyclophosphamide, dacarbazine, vinblastine, and gemcitabine. The cytotoxic agent formulations will be evaluated for loading efficiency as well as potency in cell pharmacology studies using human tumor cells (listed in Task 2) compared to non-targeted PVNs with drug cargo, targeted PVNs without drug cargo, and free drug(s) alone. These analyses will determine the optimal cargo based on loading efficiency, unloading efficiency, and maximal benefit with nuclear targeting using the cell lines from Task 2. ATP assays will be used for cell viability determinations using the Invitrogen, A22066, ATP Determination Kit. This bioluminescence assay confirms the presence of ATP in live cells, which is consumed when cells die. In the presence of ATP, luciferase will react with the substrate, luciferin, to produce oxyluciferin, AMP and light. The amount of luminescence will be measured using a plate reader at 540nm.

**Task 5. Use the targeted PVN’s loaded with an animal pharmacology-appropriate imaging reagent as described in Task 3 in a proof-of-concept study using A375 (human melanoma)-bearing nude mice.** A minimum of two permutations of targeting peptide and/or imaging cargo will be tested, each at n=3 at a dose to be determined based upon the in vitro results. Animals will be sacrificed 8 hours post-dose and tumor harvested along with normal tissues (lung, liver brain etc.). Using histological samples, the % of fluorescent cells will be determined and the ratio of targeted PVN exposure in tumor vs. normal tissue calculated.

**Task 6. Using the optimal drug-loaded targeted PVN from Task 4, execute a small proof-of-concept tumor growth inhibition xenograft study in A375-bearing nude mice.** Mice (n = 6-8 per group) will be dosed with free drug at maximum tolerated dose (MTD), at the same drug dose but loaded into PVNs as well as 2 additional groups, one at 10X this dose in PVNs and one at 1/10X in PVNs. A control group will be included as a comparator. DOX will be the drug of choice for these proof-of-concept studies, given our considerable data and experience with it. If, however, another drug from Task 4 proves to be considerably more efficacious in the in vitro studies against the melanoma cell line chosen for the xenograft study, we will use this drug instead of DOX in Task 6. The exact multiple of doses may vary based upon the cargo drug determined in Task 4. Animals will be dosed I.V. on the optimal regimen for drug alone for 3 weeks with tumor growth determined by caliper measurements.
Task 7. Develop PVNs using optimal targeting peptides and cargo of MRI agent. Using the optimal targeted peptides developed in Aim 2, we propose to load the PVN cargo chamber with contrast agents for MRI (gadolinium and manganese-based). A back up plan would be to conjugate the agent directly to RCNMV. This approach was recently applied successfully to CCMV (2). The optimal imaging contrast agent will be determined by loading efficiency (molecules/PVN) and tested via NMR in vitro. This MRI imaging PVN will not be tested in vivo until Phase II.

Task 8. Prepare a comprehensive final report for NCI. We will coordinate and communicate with all project participants in a comprehensive review process, consolidate all data, and provide a final report to the National Cancer Institute.

Figure 4. Project Timetable

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D. Related Research or R&D

*Red clover necrotic mosaic virus (RCNMV)*

RCNMV is a 36 nm icosahedral plant virus whose virion is composed of 180 copies of a 38 kDa capsid protein (CP). Each CP subunit has two surface lysines and one cysteine, adding up to 540 potential coupling sites on the surface of each virus. Evolution has conferred biological and physical properties on RCNMV that make it ideal as a mammalian cell targeting cargo delivery vehicle. First, RCNMV has evolved the capability to be stable in soil and consequently also in the mammalian circulatory system where the calcium ion concentration is in the millimolar range. RCNMV is remarkably hardy and can withstand extremes of temperature and pH, organic solvents, nuclease and protease attack and ultracentrifugation. Second, unlike liposomes, RCNMV has ion sensing properties that it has evolved to detect the conditions of the cytoplasm. In nature, RCNMV can enter plant cells that have damaged cell walls. Once inside a cell, should the calcium and magnesium concentration fall into the 100 nm range, the virion structurally changes over a period of hours into an open conformation that creates 60 pores, each with a diameter of 11-13 Å, which permits release of the viral RNA. At higher divalent cation concentrations, as found in soil, the virus is in its closed conformation, and the genomic RNA cargo is protected. We have observed that the same level of protection is provided to any added cargo. Release of encapsidated drugs is negligible in blood and other biofluids where the calcium and magnesium ion concentrations are higher than 1 mM. Therefore, RCNMV protects its cargo robustly.
yet has an exquisitely sensitive cargo release mechanism that is a key feature for its use as a nano-cargo vessel.

The RCNMV-based PVN (or simply PVN) has the following attributes as a drug delivery platform:
1. Uniform size and shape (36 nm icosahedron).
2. Large cargo capacity (up to 2570 nm$^3$).
3. Structurally defined chemical attachment sites (for cell surface targeting molecules).
4. Ability to be produced in gram quantities (plant expression scalable).
5. Robust and stable (no lipid component and stable in biological fluids).
6. Well defined chemical infusion protocol (reversible EDTA treatment).
7. Milieu-specific intracellular drug release (cooperative divalent ion effusion).
8. Replication restricted to plants (does not infect humans).
9. No inherent cell targeting capability for plant or animal cells.
10. Low immunogenicity based on presence in the food chain in temperate climates.

Comparison to other nanoparticle delivery vehicles

Alternative nanoparticle drug delivery vehicles include solid nanoparticles, liposomes, polymers, dendrimers and other viruses. Metallic, quantum dot, magnetic, or other solid materials or layered nanoparticles tend to have limited space for loading of targeting molecules and cargo and limited colloidal stability in blood. It is difficult to control release since the reporter molecules (or “cargo”) are not protected and hence are presented to biofluids at all times. For these reasons solid nanoparticles are poor drug delivery candidates. Polymer and dendrimer approaches suffer from the problem that transport of drugs often requires modification of the drug molecule, that is: attachment of a linker. Alternatively, if sequestered in a compartment, either an external trigger such as electromagnetic waves or alternatively a slowly dissolving entrapment layer must be used to release the drug. Drug release can take place anywhere: in the bloodstream; in the heart; or in healthy tissues, in general. In addition, polymeric materials tend to have a size distribution that can vary considerably from batch to batch. The production of monodisperse drug carrying polymers is an inherently difficult biomolecular problem.

Liposomes are widely used in drug delivery (3). Targeting Immunoliposomes are emerging as a method in hundreds of research studies published each year (4). The loading of liposomes, however, requires a strategy to prevent leakage. Neutral molecules tend to leak across the membrane. Charged molecules do not leak, but the kinetics of their release is very slow. Fundamentally, the major problem with liposomes is that there is no trigger for release. Plant viruses provide an alternative strategy for delivery of small molecule drugs and oligonucleotides for gene silencing and gene splicing. Protein cages such as ferritin have also been demonstrated in imaging applications as cell targeting agents (5), but loading and protection of drug cargo is problematic. Preliminary research indicates that non-enveloped icosahedral viruses also have potential for targeted cell delivery as multifunctional nanoparticles. One of the best-characterized viruses for nanotechnology applications is *Cowpea chlorotic mottle virus*, CCMV (6), which has the ability to assemble *in vitro* and a large number of surface attachment sites. Also, CPMV (*Cowpea mosaic virus*) has been investigated for use as a delivery agent (7). Expression of a peptide on the C-terminus and coat polymorphism studies (8) of *Tomato bushy stunt virus* (TBSV) demonstrates a genetic approach to preparation of targeting PVNs. These viruses have been proposed as a delivery platform based on their ease of modification, low toxicity, and lack of replication in humans. In addition to having these properties, RCNMV has the advantage of triggered release and loading of molecular cargo by infusion, as has been described. Infusion of cargo in a plant virus by a reversible process has been demonstrated only in RCNMV. In addition,
RCNMV is robust in blood and releases its cargo only when it enters a cell and has been in the cytoplasm for a number of hours.

Numerous virus vectors have been studied for cancer treatment, some with promising clinical results, yet the FDA has not approved any virus-based therapeutic agent due to concerns about toxicity that became apparent in the 1999 Gelsinger gene therapy accident (9). This incident raised concerns regarding the immune response to human adenoviral vectors. In addition to immunogenicity, Adenovirus must be genetically disabled for use as a drug or gene delivery platform. From a regulatory perspective, even the low probability event of Adenovirus recombination is sufficient to impede its development and use as a vector. The PVN platform does not replicate in any mammalian system, eliminating this concern. RCNMV can be deactivated, if necessary, so it will be incapable of replicating in plants.

**Preliminary Data Introduction and PVN Nomenclature**

Preliminary Data from Franzen, Lommel, and collaborators follow. To avoid lengthy descriptions of the PVN compositions, a nomenclature was created. The abbreviation PVN, plant virus nanoparticle, refers to RCNMV when it is used in the context of a targeted drug delivery nanoparticle. PVNs are engineered and infused with cargo in a variety of combinations. When doxorubicin (DOX) or any small molecule is infused into the PVN, its acronym is added to the left of the PVN designation, i.e. DOX-PVN (Fig. 2). If a CD46 targeting peptide is chemically attached to the exterior, its acronym is added to the right of the PVN designation, i.e. the construct: PVN-CD46. Finally, if a fluorophore, e.g. Alexa Fluor 633 (F) is used as a label on the peptide the construct is designated PVN-CD46-F. If the foregoing PVN has both a CD46 peptide and an NLS peptide, it will be designated PVN-CD46-(NLS), i.e. the second peptide in parentheses.

**Infusion of fluorophores and chemotherapeutic agents in PVNs**

When RCNMV is in an environment low in Ca$^{2+}$ and Mg$^{2+}$, channels open on the capsid surface (Fig. 3) and extend into the interior of the virion, allowing for the diffusion of charged molecules into and out of the capsids (10). Fig. 3 shows both open and closed forms. In the closed form, the virus does not contain pores. Under conditions of high Ca$^{2+}$ and Mg$^{2+}$, the RCNMV capsid is extremely stable over a wide range of solution conditions. The open form can be induced in a test tube by the addition of divalent ion chelators, such as ethylene diamine tetraacetic acid (EDTA). This condition is used to infuse small molecules into the virion. Infusion of cargo is the first step towards converting RCNMV into a PVN. The cytosol has low Ca$^{2+}$ and Mg$^{2+}$ concentration, such that the infused cargo molecules are released inside the cell, as observed by fluorescence and confocal microscopy and flow cytometry.

**Preliminary in vitro data using HeLa cells**
The Franzen and Lommel Laboratories generated HeLa cell data demonstrating that an RCNMV-based PVN enables efficient uptake and retention of dyes with positive or neutral charge, and of the drug doxorubicin (11). Second, PVNs with targeting peptides attached to the surface can be internalized when incubated with mammalian cells. Native RCNMV was conjugated via surface-exposed lysine residues to a terminal cysteine on a fluorescein-labeled peptide (notated CD46-F) from the adenovirus fiber protein, which binds the membrane complement protein, CD46. The peptides contained a terminal cysteine that was conjugated to the surface-exposed lysine residues on the RCNMV CP P-domains by means of the heterobifunctional linker molecule succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate linker (SMCC).

The fluorescent label permits monitoring of both peptide coverage on the PVN surface and the uptake of PVNs. Based on the measured fluorescence, Franzen and collaborators determined that the surface of the PVN was labeled with approximately 60 CD46-F peptides and in earlier work showed that greater amounts of peptide on nanoparticles leads to enhanced endocytosis (12). The endocytosis of these formulations was detected using fluorescence microscopy and flow cytometry. Figure 4A shows progressive movement of cell populations along the vertical (fluorescein) and horizontal (rhodamine) axes providing a measure of PVN endocytosis and intracellular release of previously quenched rhodamine, respectively. Analysis by flow cytometry indicated that PVN intracellular uptake was rapid and efficient (Figure 4B). To eliminate contributions from cellular autofluorescence, control formulations with PVN-CD46 (lacking a fluorescein label) and unmodified native PVN were assayed under the same experimental conditions. Fluorescence intensity in both cases was the same as the negative control (untreated HeLa cells), validating that increased fluorescence intensity occurred via internalization of the PVN-CD46-F.

After determining that internalization required a targeting peptide, the relationship of rhodamine (RHO) infusion to PVN internalization was determined. In another set of experiments, RHO-PVN-CD46-F uptake in HeLa cells was measured by flow cytometry after 3, 6, and 9 hours. RHO-PVN-CD46-F was internalized in more than 95% of the cells after 3 hr incubation. By contrast, the fluorescence intensity of RHO in cells was not significant at 3 h but gradually increased to 28.6% after 9 h of incubation. These and other data suggest that there is a delay of several hours between PVN internalization...
and RHO release. Control experiments with RHO-PVN showed no fluorescence above the negative control (untreated HeLa cells) over 6 h. When DOX was infused to create DOX-PVN-CD46-F, the measured level of DOX uptake was high: 1000 molecules per virion (47). Dead or dying cells were not easily measured via flow cytometry. Consequently, the effect of DOX-PVN-CD46 on cell viability was measured using an ATP assay on adherent cells. HeLa cells were exposed to: free DOX, DOX-PVN, and DOX-PVN-CD46 for 24 hours (at DOX concentrations of 0.01 - 12 μM).

Figure 5 shows the effect of DOX targeting on HeLa cells after 24 hour incubation with various formulations. Figure 5A shows normal adherent HeLa cells with no added agent. Treatment with DOXRCNMV with no targeting agent on the PVN surface, as shown in Figure 5B, leads to a slight increase in cell death at 100 pM concentration. Figure 5C shows that 100 nM DOX leads to some increase in cell death as well. The targeted delivery of DOX is shown in Figure 5D, where a 24 hour incubation with 100 pM DOXRCNMVCD46 leads to nearly complete cell death. At a loading density of 1000 DOX molecules per PVN, the effective concentration of DOX in Figure 5C is the same as that in Figures 5B and 5C. In this preliminary study with un-optimized PVN, results indicated an IC50 of 7 μM for DOX-PVN-CD46 versus 70 μM for free DOX. Cell detachment is also an early sign of apoptotic cell death. The number of adherent cells drops by 50% at a DOX-PVN-CD46-F concentration of 100 pM (data not shown). It was shown elsewhere that CD46 acts to target nanoparticles to the nucleus of HeLa cells (13). The large effect seen in Figure 5 may be expected to be attributed to nuclear targeting.

### Preliminary Data using HER-2 Targeting of MCF-7 and HeLa cells

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<thead>
<tr>
<th>Cell Line</th>
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<tr>
<td>MCF-7</td>
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<tr>
<td>MCF-7 Dox</td>
<td>RCNMVHER2-F</td>
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<td>MCF-7 Her2</td>
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</tr>
<tr>
<td>HeLa</td>
<td>RCNMVCD46</td>
<td>1 x 10^{-9}</td>
</tr>
</tbody>
</table>

Table 1. Delivery of PVNs with HER-2 and CD46 Targeting Peptides

Targeting of cell lines requires identification of a specific receptor that is overexpressed in a cancer cell line that will permit a higher concentration of the PVN to localize in that cell line. Table 1 shows flow cytometry data for the internationalization of
PVNs [expressed as in terms of 50% yield (EC50)] into cell lines with various levels of expression of the HER-2 receptor. Comparison of three variants of the MCF-7 cell line show that PVNs that are conjugated to the HER-2 targeting peptide are endocytosed at 50% yield at a lower concentration (5 x 10^{-11} M) in MCF-7/Her2 cell lines relative to the MCF-7/Dox (7 x 10^{-10} M) and MCF-7 (2 x 10^{-9} M) cell lines. Table 1 also shows that the CD46 peptide targets HeLa cells ten times more effectively than the HER-2 peptide. These differences provide the basis for discrimination of different cell lines.

**Preliminary in vivo studies**
RCNMV was evaluated *in vivo* in a study consisting of three phases by the Franzen and Lommel Laboratories and the NCSU College of Veterinary Medicine. In Phase 1, acute toxicity was determined near the solubility limit of RCNMV and well above the expected dose limit that a final formulation would take. A 2mg dose of RCNMV (70uL 28.6mg/mL) was delivered by intravenous (IV) injection via the tail vein of a Charles River Labs, Inc. Balb/c mouse. This massive dose was estimated to be more than 4 nanoparticles per cell in the animal. The mouse was monitored over the next 24 hours for any deleterious effects. At 24 hours post-inoculation, the mouse had survived and was exhibiting normal behavior: grooming, nesting, etc. Seven additional mice received injections (6 IV, 1 SubQ). In addition, 4 mice were dosed with Phosphate Buffered Saline (PBS) as a control group. The mice were observed for 21 days. There was no sign of aberrant behavior for the entire study period. Post-mortem intracardiac bleeds were done on each group and pooled for preliminary determination of immunogenicity. After evaluation by ELISA, no discernible levels of antibody presence above background were indicated and the study commenced to Phase 2.

![Figure 7. Circulating ^{131}I-labeled PVN determined by detectable radioactivity in mouse plasma.](image)

In Phase 2, immunogenicity was determined. Prior to dosage with RCNMV, a ~20ul blood sample was drawn from the lateral saphenous vein of each mouse and individual samples pooled by experimental group. On day 1, 10 animals were inoculated with RCNMV and 4 control animals were administered PBS. A 10-20ul aliquot of blood was drawn at days 0, 1, 3, 5, 7, 14, and 21 post-inoculation from the lateral saphenous vein of each animal and pooled. To assess any secondary immune response, 6 mice were administered a boost dose of RCNMV at the initial concentration after the one week time point. At the completion of the study, the final titer of the RCNMV antibodies was determined by ELISA. There was no significant amount of absorbance when compared
to a positive control (primary rabbit antibody produced by immunization with adjuvant) known to bind the RCNMV coat protein. The pooled mouse plasma of the dosed groups did not show significant absorbance above that of the PBS control group.

In Phase 3, to determine the clearance rate of circulating RCNMV in vivo, Balb/c mice were injected with virus iodinated with $^{131}$I. Aliquots of 10-20ul of blood were drawn from a lateral saphenous vein at 9 time points (pre injection, 5 (5 mice), 15 and 30 minutes and 1, 2, 3, 24, and 48 hours) to determine the RCNMV level circulating in the blood. The clearance half-life of injected, untargeted PVN (native RCNMV) is less than 5 minutes, which cannot be determined more accurately using the methods employed here. Fig. 7 shows a leveling-off effect of the residual PVN on a time scale of many hours. Urine collected in these studies contained high levels of radioactivity consistent with rapid excretion of the PVN through the kidneys. Major organs harvested for I-131 at 24 hrs. after inoculation showed no statistically significant accumulation.

E. Relationship with Future R&D
The specific aims, tasks, and timelines of this Phase I proposal are enabled by the abundance of work already completed on the characterization of the PVN as a nanoparticle delivery vehicle (see section D, immediately preceding). The goal of this Phase I proposal is to position the PVN work for the Phase II effort, specifically generating IND-quality targeted and loaded PVNs for whole body tumor imaging and therapeutic applications. An additional element of the Phase I plan is to generate a robust purification protocol for the PVNs to support both GMP/IND-enabling production as well as PVN quantity to allow the dissemination of PVNs to the academic community for research purposes.

In addition, future studies would explore combination therapy, an active area of development, because of the synergistic efficacy of two different mechanisms of action. Recent research has shown that increased efficacy of drug combinations is dependent on the ability to control the ratio of uptake of the two drugs into the cancer cell, a daunting task with free drugs. With PVN technology, however, this ratio can be fixed during the nanoparticle loading process and maintained, all the way to the nucleus of the targeted cell. A component of a possible Phase II study would be the extension of the technology to combination therapies.

F. Potential Commercial Applications
The ultimate product output initiated in this proposal is a matched pair imaging PVN and therapeutic drug PVN for cancer treatment. The imaging PVN will be used to screen cancer patients for tumor targeting selectivity and is anticipated to be valuable also in assessment of drug efficacy and for use in on-going patient monitoring for cancer progression. The therapeutic drug PVN will be used to treat cancer patients, with cell selectivity established by the imaging PVN. The proposed cell targeting technology is expected to target 50% of all solid tumors, 75% of metastasized tumors and 100% of melanoma but not healthy cells in the human body. This cell targeting will be combined with a nuclear importin to enable the PVN to evade the cancer cell defenses and deliver the therapeutic drug into the cell nucleus where the mechanisms of action of the drugs employed operate. Cancer is America’s second-biggest killer, only slightly behind heart disease. The American cancer Society estimates that in the USA there will be 1,437,180 new cancer cases in 2008 which equates to 11.5 individuals per minute during a 40 hour work week being given the news that: “You have cancer.” 565,650 individuals will die of cancer, a rate of 1.06 deaths per minute year-round. The patient pool for cancer therapies is rising with an aging global population. Business Insight calculated the global cancer drug market to be valued at $48.6B in 2006, representing a 17.9%
Increase over 2005 sales of $41.3B and that this market will grow to $74B in 2012, a 7.2% CAGR (compound annual growth rate). A breakaway targeted cancer therapeutic that truly delivers the promise of nanoparticle drug delivery has the opportunity to be a “blockbuster drug”, significantly impacting cancer patient quality of life.

A derivative product of this proposal is a generic biologic nanoparticle drug delivery system with detailed protocols for loading drugs and other agents and conjugating targeting molecules for cell selectivity. This nanoparticle drug delivery system platform technology can be used for targeted delivery of any small molecule drug and RNAi or microRNA for gene silencing or gene splicing. This platform technology would be made available to Government Research Laboratories, University Research Centers, National Cancer Centers and pharma industry partners. The availability of this nanoparticle drug delivery system will speed the development of targeted therapeutics and diagnostics for a wide range of diseases. Drug delivery is a high growth segment of the pharma industry, with key drivers being the poor solubility, poor efficacy and frequent dosing regimens of current drugs. Market forecasts project that nanobiotechnology will exceed $3 billion in 2008 with an annual growth rate of 28 percent. In a 2007 study, the Freedonia Group concluded that demand for nanotech-based medical supplies and devices in the U.S. market will exceed $110 billion by 2016. Cientifica reports that unlike other markets in which nanotechnology is merely projected to have an impact, nano-enabled drug delivery systems already represents a $3.39 billion market. According to Cientifica we are just seeing the “tip of the iceberg” as the technology shifts from polymer therapeutics to truly innovative approaches such as the NanoVector PVN. By 2012, Cientifica forecasts, the total market for nanotechnology-enabled drug delivery will rise to $26 billion representing a 37% CAGR over the next five years, and the value of these nano-enabled compounds is projected to skyrocket, reaching $220 billion by 2015.

The NanoVector strategy to enter and compete in the therapeutic drug market is to license our drugs to large pharma companies to complete the expensive Phase 3 clinical trials and to provide sales and distribution of these drugs after FDA approval. Typically, such partnering opportunities do not present themselves until completion of early Phase 2 efficacy results. Derivative nanoparticle products for the research market could be sold by NanoVector as research products through national distributors.

G. Key Personnel and Bibliography of Directly Related Work

**Dr. Oberhardt** will serve as the Principal Investigator on this project. He will:
- coordinate activities with employees, consultants and advisors;
- organize and run research meetings; and
- track project progress using Microsoft Project software.

**Dr. Steed** will coordinate the cell culture work and animal studies and ensure progress toward a formulation for future IND application. **Dr. Luft** will conduct day to day in vitro studies using PVN preparations and cell cultures to determine feasibility of product formulations. **Ms. Hu** will produce the PVNs for this study, ensuring their quality control as well as assisting Dr. Luft in the day to day experiments leading to final formulations for the animal studies and in vitro studies of encapsulated MRI contrast agent.

**Bruce J. Oberhardt, Ph.D., Principal Investigator**
President and Chief Scientific Officer, NanoVector, Inc.

<table>
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<tr>
<th><strong>Education and Training</strong></th>
<th><strong>Institution</strong></th>
<th><strong>Degree</strong></th>
<th><strong>Year(s)</strong></th>
<th><strong>Field of Study</strong></th>
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<tr>
<td>NIH Special Fellow</td>
<td>Pre/Post-doc</td>
<td>1968-71</td>
<td>Blood Diseases</td>
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<tr>
<td>Polytechnic Fellow*</td>
<td>Pre-doc</td>
<td>1966-1967</td>
<td>Neurophysiology</td>
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Polytechnic University, NY  Ph.D.  1971  Bioengineering**
Polytechnic University, NY  M.S.  1967  Bioengineering
Polytechnic University, NY  B.S.  1965  Chem. Engineering
[*NSF Sponsored  **Minors: Chemical Engineering and Electrical Engineering]

Positions and Employment
1973-1979 Sr. Scientist and Project Mgr., Technicon Instruments Corp., Tarrytown, NY
1979-1983 Mgr. of R&D, LSI Division of Miles Laboratories [Bayer], Elkhart, IN
1984-1985 Group Manager, Corp. Research Center, Becton Dickinson & Co., RTP, NC
1985-1997 Founder & CSO, Cardiovascular Diagnostics,, Inc., Raleigh, NC
1997-1999 Founder & President, Sanguinex, Inc. Raleigh, NC
1999- Pres. BJO Biomedical, LLC (Consulting / Technol. Incub. including NanoVector)

Other Experience and Professional Memberships
1. Chair, TARDC (Triangle Area Research Director’s Council)
2. Professor of Biomedical Engineering, Adjunct Faculty, North Carolina State University
   (Joint Department with the University of North Carolina-Chapel Hill).
3. Vice Chair of the Atlantis Group, LLC, a Research Triangle-based angel investment
group.  (See www.theatlantisgroup.net.)
4. Board Member: RTP Chapter, Assoc. for Corporate Growth (See: www.acgrtp.org)
5. Member, Research Triangle Regional Partnership (RTRP): Advanced Medical Care
   RTRP Cluster Group and Biological Agents and Infectious Disease Cluster Group

Selected Recognition Awards
- 1997 - North Carolina Governor’s Award for Outstanding Entrepreneurial
  Contribution
- 1996 - North Carolina Biomedical Entrepreneur of the Year Award, presented by
  Ernst and Young, NASDAQ, the Kaufmann Foundation, and USA Today.
- 1990 - Finalist, North Carolina Emerging Entrepreneur of the Year, presented by
  Ernst & Young, Inc. Magazine, and Merrill Lynch

Selected Peer-reviewed Publications (from total of 50, including abstracts)
1. Oberhardt, B. J., Lalezari, P. and Jiang, A. F. A Physicochemical Approach to the
2. Oberhardt, B.J. Thrombosis and Hemostasis Testing at the Point Of Care. American
   Journal of Clinical Pathology, (Pathology Patterns) 104, No. 4, S72-S78, 1995.
4. Oberhardt, B.J., Mize, P. D., Pritchard, C. G., Point-of-Care Fibrinolytic Tests: The

Selected Patents (from total of 181 issued worldwide)
1. Oberhardt, B.J. US 4,418,148 Multilayer enzyme electrode membrane
2. Oberhardt, B.J. and Ornstein, L. US 4,307,070 Methods and apparatuses for
   performing immunoassays
3. Oberhardt, B.J. US 5,677,133 Dry chemistry cascade immunoassay and affinity
   assay
4. Oberhardt, B.J. US 6,251,615 Cell Analysis Methods

Career Accomplishments
Performed early research in liposome, latex, and other particle-based immunologic detection systems for medical diagnostic applications; led and/or participated in industrial projects including a number of first-of-a-kind developments, such as: the first commercial flow cytometry and cytochemistry-based automated white blood cell differential analyzer used to diagnose and monitor leukemia, lymphoma, and other blood diseases [a technology used for the Technicon Hemalog D, Bayer/Technicon H Systems, and ADVIA Systems (Siemens) and still considered a “gold standard”]; other novel automated blood chemistry and cell analyzers; blood glucose sensors, blood coagulation monitors, the first microprocessor-controlled wearable insulin pump; other drug delivery systems; novel immunodiagnostic technologies, including the first electrochemiluminescent immunoassay technology; and others including “CoaguChek®”, and founded Cardiovascular Diagnostics: a successful IPO. The CoaguChek® product has been very successful in worldwide markets and has its own website; see: www.CoaguChek.com. It is of interest that the CoaguChek® system for point of care monitoring of warfarin drugs, e.g. Coumadin®, the most widely-used prescription cardiovascular drug in the US, was a technology development project initially supported by an NIH Phase I SBIR grant. Dr. Oberhardt, the PI, who patented and developed the technology, served as PI on three other NIH SBIR projects, all of which achieved Phase II awards and were developed into products that were commercialized. Prior to serving as President and CSO of NanoVector, Inc., in 2007, major focus areas included developing and consulting in multidisciplinary and disruptive technologies, involving: cell analysis; nanotechnology; medical devices; and solving problems associated with the intersection of biomedical technology and business. Career developments include technologies to help in diabetes, blood diseases, cardiovascular disease, and cancer. Products based on these technologies are currently saving patients’ lives daily.

Paul M. Steed, Ph.D.  Co-Investigator
Vice President, Research and Development, NanoVector, Inc.

Education and Training

<table>
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<th>Institution</th>
<th>Degree</th>
<th>Year(s)</th>
<th>Field of Study</th>
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<td>Post doctoral Fellow*</td>
<td>Post-doc</td>
<td>1992-93</td>
<td>Biochemistry/Drug Discovery</td>
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<td>Purdue University, IN</td>
<td>Ph.D.</td>
<td>1992</td>
<td>Molecular Biology</td>
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<tr>
<td>Lafayette College</td>
<td>B.A.</td>
<td>1986</td>
<td>Biology</td>
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[* Dept. of Molecular Biology & Enzymology, Ciba-Geigy Pharmaceuticals, Summit, NJ]

Positions and Employment

2008-Pres. VP of Research and Development, NanoVector, Inc. Raleigh, NC
2005-2008 Senior Director of Biology, Serenex, Inc., Durham NC
2004-2005 Executive Director of Preclinical Biology, Amphora Discovery Corp., Research Triangle Park, NC
2002-2004 Associate Director of Inflammation, Xencor Inc., Monrovia CA
1997-2002 Program Head, Arthritis and Bone Metabolism Therapeutic Area, Novartis, Pharmaceuticals, Summit, NJ
1995-2002 Senior Scientist/Fellow, Arthritis Biology, Ciba/Novartis Pharmaceuticals, Summit NJ
1994-1995 Senior Scientist, Molecular Biology and Enzymology, Ciba-Geigy Pharmaceuticals, Summit NJ

Professional Experience and Memberships
10 years of experience at Ciba/Novartis and 6 years in the biotech industry, primarily leading drug discovery programs/projects. Also extensive experience in drug discovery from target ID/validation through pharmacology and IND. Additionally, extensive experience in research management and strategic planning, including both small molecule and biologics-based therapeutics- an essential background for Nanovector technology. Member, American Association for Cancer Research.

**Selected Peer-reviewed Publications (from total of 35, including abstracts)**

**Patents**
1. Desjarlais JR, Filikov A, **Steed PM**, Zalevsky J, Szymkowski DE. US Pat. Appl. 200671842 Protein based TNF-alpha variants for the treatment of TNF-alpha related disorders

**Recent Accomplishments**
Most recently, led the effort at Serenex to bring SNX-5422, a selective orally-available Heat Shock Protein 90 (Hsp90) inhibitor, as an anticancer drug through IND and into clinical trials. Serenex was acquired by Pfizer in April 2008 primarily for SNX-5422.

**James Christopher Luft, Ph.D.**
Group Leader, Cell Pharmacology, NanoVector, Inc.

**Education and Training**

<table>
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<th>Degree</th>
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<td>U. of Mississippi</td>
<td>Ph.D.</td>
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<td>M.S.</td>
<td>1992</td>
<td>Microbiology/Immunology</td>
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<tr>
<td>Mislaps College</td>
<td>B.S.</td>
<td>1988</td>
<td>Biological Sciences</td>
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[* Lineberger Cancer Center, UNC Chapel Hill]

**Positions and Employment**
2008-Pres. Group Leader, Pharmacology, NanoVector, Inc. Raleigh, NC
2007 Sr. Scientist, Assay Development, Liquidia Technologies, Chapel Hill NC
2002-2006 Group Leader, Amphora Discovery Corp., Research Triangle Park NC
2001-2002 Senior Scientist, Artecel Sciences, Durham NC

**Professional Experience**
Highly accomplished Senior Biochemist and Project Leader with pharmaceutical and biotechnology experience in the areas of: 1) innovative assay development (cell-based, enzymatic, and nanotechnology), 2) initiating and maintaining collaborative relationships with academic, foundation, government, and pharmaceutical clients, 3) program management, 4) biochemical technology development, 5) research focus and prioritization, and 6) coordinating overall research operations.
Publication (selected from a total of 25 including abstracts)

Patents
PCT/US02/36317. Methods and Compositions for the Use of Stromal Cells to Support Embryonic and Adult Stem Cells

Mei Hu
Senior Associate Scientist, NanoVector, Inc.

Education and Training

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<td>Southeast U., China</td>
<td>M.D.</td>
<td>1984</td>
</tr>
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Positions and Employment
2008-Pres. Senior Associate Scientist, NanoVector, Inc. Raleigh, NC
2002-2008 Senior Associate Scientist I, Serenex Inc. Durham, NC
1999-2002 Research Specialist, Dept. of Pharmacology, U. of North Carolina School of Medicine, Chapel Hill, NC
1995-1999 Research Technician III, Dept. of Biochemistry and Biophysics, U. of North Carolina School of Medicine, Chapel Hill, NC
1991-1992 Research Assistant, Dept. of Surgery, U. of North Carolina School of Medicine, Chapel Hill, NC

Professional Experience
Senior research scientist with over 15 years experience in both industry and academia. Excellent technical skills in recombinant DNA cloning, protein extraction and purification, enzyme assays and characterization, nucleic acid analyses, tRNA purification, and established primary cell culture. Research experience has involved discovering target proteins for compound screening and IC50 determination, preparing samples for PK, PD biomarker assay. Other experience includes work with: human blood, laboratory animals and general laboratory management. Publications: total of 35, including abstracts.

Stefan Franzen, Ph.D.
Founder and Scientific Advisor, NanoVector, Inc.
Dr. Franzen is Professor of Chemistry at NCSU and has been a Member of the Duke Cancer Center, Durham, NC since 2000. Dr. Franzen has 110 peer-reviewed publications and 8 patents, including patents pending. A major portion of his research laboratory is devoted to developing methods and new approaches to drug delivery using technologies based on plant virus nanoparticles. He has studied cell targeting and formulation of nanoparticle and PVN targeting agents using both chemical and analytical means. He is one of the early developers of methods to target cancer cells with gold nanoparticles. He has ongoing research support from the Army Office of Research, the Keck Foundation, the National Science Foundation, and the North Carolina Biotechnology Center.

Steven A. Lommel, Ph.D.
Co-founder, and Scientific Advisor, NanoVector, Inc.

**Dr. Lommel is Professor of Plant Pathology and Genetics at NCSU. He is also Assistant Vice-Chancellor for Research and Graduate Programs and William Neal Reynolds Distinguished Professor.** Dr. Lommel has served on a number of journal editorial boards, including Virology and the Encyclopedia of Plant and Crop Science and has 80 peer-reviewed publications and 3 patents, including patents pending. The Lommel laboratory is devoted primarily to the study of the Red Clover Mosaic Necrotic Virus (RCNMV) which forms the basis of the NanoVector Technology. Study of RCNMV plant pathology and genetics is an ongoing effort in the Lommel laboratory, using techniques developed by Dr. Lommel and collaborators. He has ongoing support from the National Science Foundation and the North Carolina Biotechnology Center.

**Section G. Bibliography**


**H. Subcontractors/Consultants**

**Dr. David J. Adams, Ph.D. is Associate Professor of Medicine and Interim Co-Director of the Pharmaceutical Research Services Shared Resource, Duke University Medical Center.** Dr. Adams has 51 peer-reviewed publications and 3 patents, including patents pending. He has ongoing support from the National Institutes of Health and private industry sponsored research. Dr. Adams has a broad background in anticancer drug discovery and development that spans eleven years in the pharmaceutical industry and another eleven years up to the present at Duke University Medical Center. He also possesses extensive laboratory skills that include cell, tissue
and animal models as well as numerous analytical techniques associated with cancer research. Dr. Adams will consult on this proposed project to review overall data, including the cytotoxicity and animal data developed with the PVN drug formulations. He will also be available as a resource in the cell culture work.

**Piedmont Research Center, a division of PPD, Inc.,** will serve as vendor for performing human tumor xenograft studies in mice and the supporting pharmacodynamic, biomarker and pharmacology studies. The exact cost of Tasks 5 and 6 will be dictated by the ultimate cargo and targeting peptides determined in prior Tasks, because the dose and regimen will be unique to the drug cargo and targeting PVN combination. Dr. Paul Steed, NVI’s VP of R&D, who has worked on projects with Piedmont for the past 6 years, has consulted with Piedmont on this topic, concluding that our cost estimate is as accurate as possible at this point in time. If the costs exceed this estimate, NVI will absorb the additional expense.

**I. Facilities and Equipment**

Except for the planned animal studies, the minimal work involved in harvesting plants from the greenhouse, and possible analytical services at NCSU that may be required, the proposed research will be conducted primarily at NanoVector’s laboratory at 617 Hutton St., Suite 107, Pylon Commercial Park, Raleigh NC, 27606-6301. This facility is located a few minutes from the North Carolina State University (NCSU) Centennial Campus and close to the NCSU greenhouse complex where NanoVector is harvesting virus-infected plants under an agreement with the University. The Company’s facility has 887 square feet, including one wet laboratory with a fume hood, an inner office within the lab, and an external office of 122 square feet. Additional common and shared areas are available for meeting space. This facility will be essentially 100% available for this project. The company has available for this research basic laboratory equipment, including: two microscopes (one with a video camera and monitor) suitable for cell-related work, two computers, centrifuges, a refrigerator, a spectrophotometer, assorted glassware, pipettes, tissue culture supplies (media, serum, and plastic ware), and chemicals and reagents and small equipment. Capital has been set aside to install a new laminar flow hood, a CO₂ incubator, and a small autoclave, as well as a new plate reader (a BioTek PowerWave XS Spectrophotometer plate reader from Fisher Scientific) at this location. The company also plans to acquire other new laboratory equipment for this facility, some of which is relevant to the proposed project, including: an additional inverted microscope; a fluorescent microscope; a new precision analytical balance, and other miscellaneous pieces of equipment and supplies that will be in place at that time the proposed project would be undertaken. The proposed new virus purification process and PVN preparation for this project would also be conducted at this facility. If needed, other major pieces of equipment are available at NCSU (through user or service fees) including: a confocal microscope, an electrospray and MALDI-TOF system, and a flow cytometer (Becton Dickinson, FACScalibur) equipped with an argon laser (488 nm) and Cell Quest software. Flow cytometry samples can be run for a user fee of $20 each. The fully-equipped laboratories of Professor’s Franzen (biophysical chemistry) and Lommel (plant virology) are also available under the company’s sponsored research agreement, should any troubleshooting beyond the normal course of research become necessary. The planned animal studies will be conducted at Piedmont Laboratories, a division of PPD, Inc., in Morrisville, NC (20 minutes away) via a service agreement. Piedmont is a world class, fully-equipped animal facility specializing in pharmaceutical product R&D for IND submission.
Appendix: Vertebrate Animals

Piedmont Research Center (a Division of PPD, Inc.), Proposed Studies:

Piedmont Research Center is the vendor of choice for performing human tumor xenograft studies and the supporting pharmacodynamic, biomarker and pharmacology studies, and is located in close proximity to NanoVector (see http://www.ppdi.com/services/preclinical/preclinical_eval/xenografts.htm). As a vendor/CRO, by definition the proposed studies (as all studies in the facility) will comply with all State and US regulations regarding animal care and experimental design.

1) Xenografts will be initiated from human A375 melanoma tumors maintained in athymic nude mice. Each test mouse will receive a 1 mm³ tumor fragment implanted subcutaneously(s.c.) in the right flank. Tumor sizes will be monitored as the average size approached 80 to 120 mm³. On Day 1 of the study mice will be placed into groups, the sizes of which depending on the study designs outlined in Tasks 6 and 7. Individual tumor sizes will range from ~60 to ~220 mm³ and group mean tumor sizes predicted to be ~85 to ~100 mm³.

2) The number of mice used in this proposal will permit sound formulation design for the effective delivery of targeted PVNs. The order of testing, outlined in Tasks 6 and 7, will minimize the number of animals required by focusing on key proof-of-concept endpoints. This approach will keep the number of animals required in subsequent phases of the study to a minimum. The strains of mice used this proposal were dictated by previously published studies outlining the use of xenograft mice.

3) As an in vivo pharmacology vendor serving the cancer research/pre-clinical development worldwide, Piedmont Research abides by all international, United States and North Carolina regulations regarding animal care and husbandry.

4) Piedmont Research abides by all international, United States and North Carolina regulations regarding handling.

5) Similarly, Piedmont Research abides by all international, United States and North Carolina regulations regarding euthanasia for human tumor-bearing animals in use for testing cancer therapeutics.
October 30, 2008

Paul Steed, Ph.D.
Vice President, R & D
NanoVector, Inc.
P. O. Box 98385
Raleigh, NC 27624

Dear Paul:

This letter is to indicate the willingness of Piedmont Research Center (PRC) to provide services as a vendor in support of NanoVector’s proposed project entitled “Multifunctional Therapeutics using Engineered Plant Virus Nanoparticles”. We understand that your project is in response to NCI solicitation No. PHS 2009-1, Topic No. 267 and that the Principal Investigator is Bruce J. Oberhardt, Ph.D. Specifically, PRC has capabilities to conduct preclinical evaluations of nanoparticle drug carriers in experimental tumor models, comprising syngeneic murine tumors and human tumor xenografts. PRC has experience conducting widely accepted efficacy protocols and tissue sampling protocols for the evaluation of investigational cancer therapies.

Now in its tenth year, PRC was acquired by PPD Inc. in 2002 and continues to operate its AAALAC-accredited research facility in Morrisville, NC, a short drive from Raleigh, the airport, and local research campuses. This proximity continues to promote investigator interaction as needed. We routinely support oncology R&D projects in the pharma industry, including evaluation of novel drug carriers and studies to support clinical development plans, as well as projects with academic investigators. As the proposed project moves forward, PRC will be able to offer additional preclinical support services such as biomarker evaluation in xenograft tissue. I am confident that we can more than adequately meet your needs and NCI needs for this particular project.

We look forward to a favorable funding decision on this project, which promises to open additional new imaging and treatment options for patients with cancer.

Sincerely,

[Signature]

Steadman D. (Chuck) Harrison, Ph.D.
Senior Research Director
DUKE UNIVERSITY MEDICAL CENTER
Department of Medicine

October 29, 2008

Bruce Oberhardt, Ph.D.
Chief Scientific Officer
NanoVector, Inc.
PO Box 98385
Raleigh, NC 27624-8385

Dear Dr. Oberhardt:

I would like to express my strong interest in the proposal developed by NanoVector, Inc. to develop further the nanoparticle drug delivery platform licensed from NC State University. Using the same targeting for detection and treatment of a tumor could ensure tumor-specific drug delivery and maximize therapeutic benefit. It is my understanding that the goal is to develop an optimized drug-carrying, targeted therapeutic and progress into IND-enabling studies. In the proposed Phase I studies, cell cultures from human cancers: breast, lung, prostate, colon, and melanoma will be tested in vitro, and feasibility in a mouse model studied using a melanoma xenograft.

I am excited about the possibilities of using the drug-encapsulating plant virus for intracellular targeting and drug delivery to the nucleus in the cancer cells employed in the proposed study, especially the cells known to exhibit MDR.

I will gladly serve as a consultant to this project to review the data obtained and provide interpretation for future product applications in oncology. I will provide guidance to the excellent team you have assembled on the cell culture work that I understand will be performed at NanoVector. In my view, 5% of my time should be adequate for this 6-month Phase SBIR contract. I will set aside this time when the project is scheduled.

If I can be of further help prior to the start of this project or if there are any questions that I may be able to answer, please let me know.

Sincerely yours,

David J. Adams, Ph.D.
Associate Professor of Medicine
Interim Co-Director Pharmaceutical Research Shared Resource
Department of Medicine
DUMC 2638
Durham, NC 27710