PRINCIPAL INVESTIGATOR: Annemarie Shibata, Ph.D.


Proposal Abstract:
Approximately 42 million people are infected with human immunodeficiency type-1 (HIV-1) worldwide. While antiretroviral therapy (ART) has significantly reduced HIV-1 disease morbidity and improved life expectancy, drug expenses, treatment failures, dosing complexities, and limited global access have prevented the full utility of ART. Dosing regimens that require multiple daily dosing with diet considerations and ART side effects have reduced the achievement of long-term HIV-1 suppression in infected patients. Novel drug carrier systems, such as nanoparticles, are promising tools that may help overcome pharmacokinetic obstacles and provide successful universal therapy. Previous work in the lab has determined that ART nanoparticles are taken up by and are non-toxic to immune cells targeted by HIV-1. This proposal is designed to analyze the ability of ART nanoparticles to reduce HIV-1 viral replication rate and HIV-1's ability to infect in these immune cells using defined cellular model systems.
I. Statement of Problem/Purpose

Each year, more than 18,000 people in the United States die from Acquired Immune Deficiency Syndrome (AIDS) as a consequence of human immunodeficiency type-1 (HIV-1) infection. Currently, more than one million people are living with HIV-1 in the United States and an estimated 56,300 Americans are infected annually. Globally, more than 25 million people have died since the epidemic began, 2 million people die annually and approximately 42 million people are living with HIV/AIDS. While this is a global health issue, 74% of infected individuals live in developing countries. Additionally, of the 14,000 new cases of HIV diagnosed each day, 95% are isolated to developing countries and half of those occur among people between the ages of 15 and 24. While antiretroviral therapy (ART) has reduced HIV/AIDS morbidity rates and increased life expectancy, drug expense, complex treatment protocols, dietary requirements for drug effectiveness, treatment complications, and difficulties associated with global distribution have limited the usefulness of ART. Consequently, continued research directed at developing drug therapies designed to work within the medical limitations presented by developing countries are of great importance. We have postulated that nanoformulated drug delivery systems, or nanoparticles, can address the challenges of ART.

II. Significance of Problem

Nanotechnology is likely to become more commonplace in the delivery of drug therapy as pharmaceutical innovation continues. Since 2005, 1721 English language articles related to nanotechnology and drug delivery are identified on PubMed, a scientific search engine. Increasing interest in this field of drug research demonstrates the promise of nanotechnology-derived drug delivery paradigms. Given that ART-NPs demonstrate sustained, slow drug release and that immune cells targeted by HIV can engulf ART-NPs, ART-NPs appear to be a viable method to overcome some of the physiochemical problems associated with anti-retroviral medicines. While our preliminary data is intrinsically interesting, the potential of this work was recognized by the wider scientific community at a national retroviral meeting in Boston. Our CROI received significant attention and was incorporated into a press release that can be viewed at Creighton University's AlumWire, March 2008, No. 5, Vol. 11 entitled "CU Researchers Create Slow-Release HIV Therapy Drugs Lopinavir, Ritonavir, and Efavirenz. At the moment, PubMed and other scientific search engines indicate that no other publications than our own presents evidence for successful combination ART fabricated onto nanoparticles. Further, the federal research budget, while decreasing in some areas of research, has increased or remained constant for HIV/AIDS related work at approximately $2.6 Billion. This suggests that the interest in antiretroviral therapy development is not only of scientific interest but is also recognized politically as an important research focus. Continued work, providing available funding and resources, will yield a clearer understanding of the usefulness of ART-NP and is likely to put Creighton at the forefront of nanoparticle drug delivery research with continued development of Antiretroviral Nanoparticle Development Program at Creighton University.
III. Summary of Pertinent Literature

Nanoparticles are stable, solid colloidal particles consisting of macromolecular material ranging in size from 10 to 1,000 nm. Drugs can be adsorbed on the particle surface or entrapped or dissolved in the particle matrix. Additionally, based on nanoparticle size, shape, and composition, drugs with variable biochemical properties absorbed onto nanoparticles can be readily taken up within cellular cytoplasmic vesicles. Nanoparticle drug carrier systems provide a mechanism for enhanced drug bioavailability that is both stable within cells while providing for controlled drug release. Previous nanoparticle research has shown that nanoparticles carrying a single antiretroviral drug can reduce HIV-1 infectivity. However, the most successful ART strategies for human treatment utilize a combination of antiretroviral drugs. We theorized that if one ART drug can be used to fabricate NPs, a combination of ART agents fabricated onto NPs (ART-NPs) could significantly improve the pharmacodynamics and pharmacokinetics of ART. Our preliminary work demonstrated that lopinavir, ritonavir, and efavirenz, three commonly used drugs for ART, can be packaged into a nanoparticle drug delivery system (ART-NPs). Using separate cellular viability assay systems, we determined that cells exposed to ART-NPs survive as well as control cells and function normally over a 28 day period of observation. In vivo experiments involving the injection of ART-NP into mice demonstrated that sustained levels of ART drug could be collected from tissue.

Cellular targets for HIV-1 infection include CD4+ T lymphocytes and cells of the mononuclear phagocytic system, monocytes and differentiated macrophages. Since T cells and macrophages in a mature, immunologically active state can be productively infected with HIV-1 altered cellular functions in the macrophage population may contribute to the development and clinical progression of AIDS. For example, accumulating evidence shows that T cells and macrophages are vectors for the transmission of HIV-1. The placental macrophage is likely to be the primary cell type responsible for vertical transmission of HIV-1 from mother to infant. Further, the ability of HIV-1 to infect the mucosal lining relies upon its ability to infect macrophages. Because of the important role of T cells and monocytes/macrophages (Mo/Mac) in the pathogenesis of HIV-1, fully effective ART must react with these cells. As phagocytic cells that effectively incorporate particles into cytoplasmic vesicles found on the outside of the cell, T cells and macrophages are useful targets for nanoparticle drug therapy and are a primary focus of this proposal.

IV. Research Question and Hypothesis

This proposal addresses the ability of nanoparticle carrier systems coated with combination ART, rather than a single ART drug, to both reduce HIV-1 replication rate in infected cells and protect cells targeted by HIV-1 from infection. Unlike previous work, cellular assays developed in my laboratory and combination ART nanoparticles coated with ritonavir (RTV), lopinavir (LPV) and efavirenz (EFV) (designed by a collaborator Dr. Destache, School of Pharmacy at Creighton University) provide a mechanism by which to assess drug carrier systems delivering multiple ART drugs. These studies investigating RTV, LPV, and EFV...
coated nanoparticles (ART-NPs) are particularly interesting because these three drugs are currently being administered in clinical settings for traditional ART therapy. The hypothesis of this proposal is that ART-NPs are able to transport multiple, clinically relevant, antiviral drugs to macrophages and T cells to reduce or eliminate HIV-1 infection.

Outcomes of these studies are will increase our understanding of the viability of using ART-NPs as an immunotherapy that potentially reduces the required dose, minimizes toxicity and side effects, and improves the delivery of antiretroviral drugs in humans. Improvement of such pharmacodynamics could potentially launch cheaper, easily distributed and regulated highly active anti-retroviral therapy (HAART) to HIV-positive patients in both the United States and developing countries.

V. Design and Methods

Our preliminary data demonstrates that ART-NPs deliver sustained, slow release of ART drugs in vitro and in vivo. Further, in a cellular system, adult human macrophages were shown to readily phagocytose ART-NPs using direct immunofluorescence and FLOW cytometry of ART-NPs conjugated to the hydrophobic fluorescent dye, 6-coumarin. Cellular viability of macrophages exposed to ART-NP was not significantly different than controls providing evidence that ART-NPs are candidates for drug delivery to HIV infected macrophages. Taken together, these data suggest that ART-NPs are a viable, novel mechanism for antiretroviral drug therapy and warrant continued investigation that specifically addresses the ability of ART-NPs to reduce and/or prevent HIV-1 infectivity.

A. Design of Study

The current proposal is designed to complete the final procedures/experiments for a manuscript presenting data on the efficacy and ability of ART-NP to attenuate and/or prevent HIV-1 infectivity in adult human immune cells.

Specific Aim: To determine whether the ART NP will produce rapid and sustained attenuation of or resistance to viral replication compared to free drugs and blank NP using in vitro (outside of the animal) model systems. Two experiments paradigms will be used to determine the ability of ART-NPs to reduce HIV-1 levels in previously infected cells and to determine whether ART-NPs can inhibit HIV-1’s ability to infect immune cells.

B. Procedures/Experiments

Experiment 1: Determination of ART-NPs ability to reduce HIV-1 infectivity in adult T cells and macrophages.

HIV-1 infected T cells and macrophages and uninfected control cells (immune cells targeted by HIV-1 infection) will be treated with four conditions:

1. Free ART drugs RTV, LPV, and EFV (drug not on nanoparticles (NPs))
2. ART-NPs (formulated by Dr. Chris Destache),
3. Blank NPs
4. PBS (phosphate buffered saline, no treatment control)

*In vitro* Cellular Assay: Adult human H9 T cells (purchased from ATCC, Washington DC) and freshly elutriated adult human macrophages (purchased from Advanced Biotechnologies, Columbia, MD) will be cultured in DMEM supplemented media as indicated by supplier. HIV-1 infected cells will be treated with HIV-1 virus for 24 hours prior to application of test conditions listed above. Uninfected and HIV-1 infected cells will be harvested at 24hrs, 4 days and 7 days following application of free ART drugs, ART-NPs, Blank NPs and no treatment before harvesting for analysis (described below) to determine HIV-1 replication rates in our four conditions.

Experiment 2: Determination of ART-NPs ability to inhibit or block T cell and B cell susceptibility to HIV-1 infection.

*In vitro* Cellular Assay: Adult human H9 T cells (purchased from ATCC, Washington DC) and freshly elutriated adult human macrophages (purchased from Advanced Biotechnologies, Columbia, MD) will be cultured in DMEM supplemented media as indicated by supplier. Cultured cells will be pretreated for 24 hrs with free ART drugs, ART-NPs, Blank NPs, and PBS (no treatment control) before HIV-1 infection. H9 T cells and macrophages for all four conditions will then be exposed to HIV-1 and infection levels will be assessed. Control cells will be not be exposed to HIV-1. Control cells and HIV-1 exposed cells will be harvested at 24hrs, 4 days and 7 days following application virus to determine the level of HIV-1 infection in our four conditions.

Note: Cells are grown in their appropriate tissue culture media in tissue culture incubators in Dr. Mike Belshan’s P2 laboratory which is designed and approved for viral infection experimentation in the Department of Medical Microbiology and Immunology on Creighton University campus.

C. Data Analysis

For both experiment, levels of HIV-1 in cells will be determined. We will harvest cells at the time points outlined above and determine levels of HIV-1 using the three methods of data acquisition and analysis.

*p24 ELISA Assay*: A 24 kilodalton protein (p24), immunologically distinct from proteins in most other retroviruses, is a major structural core component of HIV-1. Mouse monoclonal antibodies with high specificity and affinity for this viral protein are used in an enzyme-linked immunosorbent assay (ELISA) to detect levels of HIV-1 in cellular samples. These kits are commercially available. T cells and macrophages from Experiments 1 and 2 will be collected. Cells will be disrupted and their proteins collected. Protein samples from these cells will be applied to p24 ELISA assays and used to determine levels of HIV-1 inside cells. Samples will be run in triplicate and an ELISA plate reader equip with will be used to analyze data. A Pearson correlation coefficient and oneway ANOVA will be used to determine significance of the data in our four conditions.
Subcellular Fractionation Experiment and Western Blot Analysis: Following infection viral proteins are often localized to specific compartments within cells. These compartments include the nucleus, mitochondria, plasma membrane, and vesicles. HIV-1 and its associated proteins have been localized to the cytoplasm, nucleus and vesicles of infected cells. Subcellular fractionation of cells is a useful technique for isolating proteins in specific compartments for further analysis. Subcellular fractionation of uninfected and HIV-1 infected cells is a useful method for investigating the mechanism by with ART-NPs target HIV-1 inside cells. Thermo scientific subcellular fractionation kits will be purchased and methods followed to isolate separate cellular fractions of T cells and macrophages from experiments 1 and 2 outlined above. Total protein collected in each of these fractions for T cells and macrophages for all conditions will be analysed for specific HIV-1 protein levels using SDS-PAGE gel electrophoresis (separates proteins by size and charge) followed by western blot analysis. Western blot analysis employs the use of antibodies to detect specific proteins transferred from SDS-PAGE gels onto membrane. Antibodies to p24 and gp120, both specific proteins for HIV-1, will be used to detect HIV-1 levels in specific cellular compartments. From these assays we expect to be able to determine not only whether ART-NPs reduce HIV-1 replication and ability to infect cells but also where HIV-1 is concentrated in cells and how well ART-NPs reduce this concentration. Western blots will be performed in triplicate and chemiluminescence will be used for detection. Relative intensity of protein bands as detected by antibodies will be analyzed using a BIORAD Geldoc system and software. One-way ANOVA will be used to determine difference between groups.

D. Schedule for completing the project and goals for future support

The proposed summer project is part of an ongoing research program. Previous funding has provided the resources necessary to begin development of the nanoparticle project at Creighton University. Continued funding through internal grants such as this summer award are vital to completion of this most recent manuscript. It is anticipated that the objectives of this specific aim will be accomplished by the end of the summer 2011 and that the manuscript will be completed and submitted for review in early September of 2011. The goal for submission of this work is particularly important because it will serve as support for my R15 application to the National Institute of Allergy and Infectious Disease within NIH in October of 2011 (awards are for two years, 275,000/yr). R03 or R21 NIH grant are also possible funding opportunities. Future, private funding will be sought through organizations such as amfAR, or the Foundation for AIDS research grants which target innovating basic science research projects.
VI. References
12. Takeshita, S, Belgum, T, Destache, CJ, Shibata A Investigations of the efficacy of nanoparticle antiretroviral drug delivery systems. 2010. 119TH Annual Meeting of the Nebraska Academy of Sciences, Lincoln,NE,
## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2. Photocopy this page or follow this format for each person.

### NAME
Annemarie Shibata, Ph.D.

### POSITION TITLE
Assistant Professor

### EDUCATION/TRAINING

<table>
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<tr>
<th>INSTITUTION AND LOCATION</th>
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<tr>
<td>Creighton University, Omaha, NE</td>
<td>B.S. (Magna Cum Lauda)</td>
<td>1992</td>
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<td>Colorado State University, Ft. Collins, CO</td>
<td>Ph.D.</td>
<td>1997</td>
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### RESEARCH AND PROFESSIONAL EXPERIENCE

4/97-6/99 Postdoctoral Research Associate, Eppley Institute for Research in Cancer and Allied Disease, University of Nebraska Medical Center (UNMC), Omaha, NE. (Laboratory Supervisor, Dr. Thomas Smithgall)

7/99-5/01 Postdoctoral Research Associate and Chief, Neuroregeneration Program, Center for Neurovirology and Neurodegenerative Disorders (CNND), Department of Pathology and Microbiology, UNMC, Omaha, NE (Laboratory Supervisor, Dr. Howard Gendelman)

8/02-5/05 Resident Assistant Professor, Biology Department, Creighton University, Omaha, Nebraska (Department Chair, Dr. John Schalles)

8/06-present Assistant Professor, Biology Department, Creighton University, Omaha, Nebraska (Department Chair, Dr. Mark Reedy)

### Grant Support:

12/94-3/97 NIH Predoctoral Fellowship FBM092, *Inhibitory Regulation of Dendritic and Axonal Growth Cones*

4/97-5/98 NIH Research Training Fellowship, received from the Eppley Institute for Research in Cancer and Allied Disease

6/98-3/2000 NIH Postdoctoral Fellowship 1 F32 Ca 79194-01, *Regulation of Small GTPases by the c-Fes Tyrosine Kinase*

5/07-8/07 Summer Faculty Research Fellowship, Creighton University, *Neurotrophic properties of microglia: implications for neuronal survival and regeneration, $5000.00*

3/07-present Creighton University and BD Biosciences Flow Cytometry Seed Grant. *Use of FLOW cytometry to investigate intracellular signaling in primary neurons following damage and exposure to stimulate microglia, $500.00*

5/08-4/09 Research Initiative Grant, Creighton University, *Determination of the Efficacy of Nanoparticle Driven Antiretroviral Drug Delivery Systems Using Cellular Model Systems, $10,000*

2008-2009 1R15AI076039-01A1 National Institute of Allergy and Infectious Diseases, National Institutes of Health Co-investigator, Awarded to primary investigator,
Dr. Chris Destache, *Pharmacology of Antiretroviral Nanoparticle Micelles.* $224,673.

2008-2013 NIH/UMNC/INBRE, *Intracellular and epigenetic mechanism underlying neurotrophic properties of activated microglia,* subcontract primary investigator, $31,000 per year

**Professional Memberships**

1996-present Society for Neuroscience  
2009-present Sigma Xi  
2007-present Nebraska Academy of Sciences  
2009-present Midlands Society for Neuroscience

**Honors and Awards**

2007 Creighton University and BD Biosciences Flow Cytometry Seed Award, $500  
2007 Summer Faculty Research Fellowship, Creighton University, $5000  
2006 Health Future Foundation Program Award, Creighton University, $77,877  
2000 Invited Speaker, Mini-Medical School, University of Nebraska Medical Center  
1998-2000 NIH Postdoctoral Fellowship  
1997-1998 Eppley Cancer Institute Postdoctoral Fellowship, Univ. of Nebraska Medical Center  
1995-1996 Travel Award, Program of Molecular, Cellular and Integrated Neurosciences, Colorado State University  
1994 Research Scholarship, Program of Molecular, Cellular and Integrated Neurosciences, Colorado State University  
1993-1997 NIMH Predoctoral Fellowship, Colorado State University  
1988-1992 Centennial Scholarship, Creighton University, Omaha, NE

**Publications:**

Destache CJ, Belgum T, Christensen K, Shibata A, Sharma A, Dash A. Antiretroviral release from PLGA nanoparticles in mice. (Submitted to Journal of Antimicrobial Chemotherapy)


Abstracts:


Yanov D, Kratochvil J., Shibata A. (2007) Establishment of Rat Pheochromacytoma Cell Culture for Experiments Investigating Neuronal Differentiation Biology Department Student Research Colloquium. Creighton University, Omaha, NE.

Lorenzen K., Shibata A. (2007) Development of an in vitro model system to assess the neurotrophic and neurogenic properties of stimulated microglia. Ferlic poster presentation, Creighton University, Omaha, NE.


Lorenzen K., Shibata A. (2008) Activated microglia stimulate neuronal survival and
2. Proposed Budget and Justification

Total Proposed Cost: $5,000+
Budget Request: $5,000

Itemized Estimated Cost:

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<td>P24 ELISA Assay Kit, p24</td>
<td>$2400.00 (4 kits/$600 each)</td>
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<td>Subcellular Fractionation Kit</td>
<td>$1200.00 (3 kits/$400 each)</td>
<td>Thermo Scientific</td>
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<tr>
<td>Western Blot Antibodies, p24 and gp120</td>
<td>$1800.00 (6 samples needed at $300 each)</td>
<td>R&amp;D Systems</td>
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Justification:
Total proposed costs include resources to culture uninfected and infected human T cells and macrophages and prepare cellular material for analysis by western blot and microarray technology. These costs will be covered by the primary investigator. Equipment for cell culture has been purchased through Dr. Shibata.
start up funds and additional incubator facilities for infected cells are housed in Dr. Mike Belshan’s lab. Free ART drug and NPs for all conditions are provided by Dr. Chris Destache in the School of Pharmacy. Supplies and materials requested in this application are specific for projects designed and implemented in Dr. Shibata laboratory. Current grant funding does not cover this project, and the summer award funds would be used to complete a manuscript necessary for an R15 application to be submitted in October of 2011.