University of Pittsburgh

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The University of Pittsburgh received $8,975,120 in formula funds for the grant award period January 1, 2009 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Small Molecule Inhibitors of HIV Nef Signaling - The project focuses on discovery of small molecules to target a unique human immunodeficiency virus type 1 (HIV-1) protein, called Nef, which is essential for progression from a state of HIV infection to full-blown acquired immune deficiency syndrome (AIDS). Using *in vitro* and cell-based screens, the investigators aim to identify two classes of drug-like compounds with potent activity against HIV replication. These studies could eventually provide new tools for understanding Nef interactions with host cell proteins and identify novel lead compounds for anti-HIV therapeutics.

Duration of Project

1/1/2009 - 3/31/2011

Project Overview

The HIV-1 accessory protein Nef is essential for high-titer viral replication and AIDS progression. Nef has no known catalytic activity and functions by interacting with multiple host cell signaling proteins, including the Src family kinases Hck, Lyn, and c-Src. Recently, we established *in vitro* and cell-based screens to identify small molecules that target the Nef:Hck complex. These screens identified two classes of drug-like compounds with potent activity against HIV replication *in vitro*, providing the first demonstration that small molecules targeting an HIV accessory protein-host cell protein complex have anti-HIV activity. This project will pursue these compounds based on their activity against a broad range of HIV strains, including strains resistant to current antiretroviral drugs. We will also explore synergy of these Nef-directed compounds with established anti-HIV therapeutics and investigate their molecular mechanism of action. Finally, we will test the hypothesis that the Nef oligomerization interface is a rational target for Nef-directed anti-HIV therapy. Previous studies have established that Nef oligomerization contributes to host cell kinase activation as well as viral and immune receptor downregulation. Small molecule inhibitors of Nef oligomerization may interfere with these critical Nef functions *in vivo*, providing new scaffolds for anti-HIV drug development.

The project's specific aims are:

Aim 1. Explore the utility and mechanism of action of newly discovered diphenylfuropyrimidines (DFP compounds) and benzenesulfonamide diaminoquinoxalines (Q compounds) as Nef-directed anti-HIV agents.

Aim 2. Test the hypothesis that the Nef oligomerization interface is a rational target for anti-HIV therapy.

Principal Investigator

Thomas E. Smithgall, PhD
William S. McElroy Professor and Chair, Microbiology and Molecular Genetics
University of Pittsburgh School of Medicine
E1240 Biomedical Science Tower
Pittsburgh, PA 15261

Other Participating Researchers

Jerrod Poe, PhD; Dora Pene-Dumitrescu, PhD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

Nef is one of six accessory proteins unique to HIV and essential for AIDS progression. HIV Nef works through multiple host cell signaling pathways in HIV-infected cells. One group of these proteins, the kinases, normally transmits signals for cellular growth and survival. Previous work from our group has established that HIV Nef binds tightly to several members of the Src kinase family, leading to constitutive kinase activation. In collaboration with the University of Pittsburgh Drug Discovery Institute, we have performed high-throughput screens of chemical libraries to identify selective chemical inhibitors of the Hck:Nef complex. These screens led to the identification of two novel classes of HIV replication blockers. In this project, we will investigate the specific mechanism of action of these compounds in HIV-infected cells (Aim 1). In addition, we will launch a new screening campaign to identify compounds that interact directly with the Nef protein (Aim 2). The expected outcomes and benefits of these studies are at least two-fold: First, these compounds will provide exciting new chemical probes for exploring the link between host cell kinases and Nef in HIV pathogenesis, which is a poorly understood area of HIV biology. Second, these compounds represent early-stage leads for the development of a completely new strategy for AIDS chemotherapy.

Summary of Research Completed

Aim 1. Nef is an HIV-1 accessory protein essential for AIDS progression and an attractive target for drug discovery; however, lack of a catalytic function makes Nef difficult to assay in chemical library screens. To circumvent this problem, we developed a high-throughput assay for inhibitors of Nef function by coupling the protein to one of its host cell binding partners, the Src-family

kinase (SFK) Hck. Hck activation is dependent upon Nef in this assay, providing a direct readout of Nef activity *in vitro*.

In a pilot screen with this assay, we identified DFP analogs as strong inhibitors of Nef-dependent Hck activation. These compounds also exhibited remarkable antiretroviral effects, blocking Nef-dependent HIV replication in cell lines where HIV replication is highly dependent on Nef. These studies, which were conducted prior to this reporting period, identify the first examples of lead compounds directed against HIV-1 Nef and one of its host cell binding partners to exhibit antiretroviral activity.

During the current reporting period, we extended these pilot studies to include a series of primary Nef proteins derived from HIV-1 isolates representative of all major HIV-1 subtypes responsible for the AIDS pandemic. First, we showed that Nef proteins from HIV-1 subtypes A1, A2, B, C, F1, F2, G, H, J, and K strongly activate Hck and the other Src-family members Lyn and c-Src. These results demonstrate, for the first time, that SFK activation is a highly conserved property of primary M-group HIV-1 Nef isolates. Hck activation by each of the Nef proteins was also inhibited by the DFP compounds. To determine whether DFPs also exhibited broad-spectrum Nef-dependent antiretroviral activity, we constructed chimeric forms of the HIV-1 strain NL4-3 expressing the same 10 primary Nef alleles. The infectivity and replication of these Nef chimeras was indistinguishable from that of wild-type NL4-3 in three distinct cell lines (MT2, U87MG, and CEM-T4). Importantly, the DFP compounds potently inhibited replication of all 10 chimeric forms of HIV-1 in both U87MG and CEM-T4 cells in a Nef-dependent manner. These results demonstrate that activation of Hck, Lyn, and c-Src by Nef is highly conserved among all major clades of HIV-1 and that selective targeting of this pathway uniformly inhibits HIV-1 replication. A manuscript describing these findings has been submitted for publication to *PLoS Pathogens.*

We previously described a high-throughput screening (HTS) campaign that yielded 343 confirmed hits with IC_{50} values (concentration of an inhibitor where the response is reduced by half) for Nef-induced Hck activation of less than 20 μM (overall hit rate of about 0.15 percent). Each of these compounds was tested for activity against Hck alone in an automated counterscreen, and comparison of the two datasets identified 67 compounds in which kinase inhibition in the presence of Nef was at least three-fold higher than when compared to Hck alone. In this reporting period, each of these compounds was tested for Nef-dependent anti-HIV activity in both CEM-T4 T cells and the U87MG astroglioma cell line. Five compounds with chemically diverse structures showed antiretroviral activity, two of which were remarkably potent, blocking HIV replication with IC_{50} values in the 100 nM range. Molecular docking studies predict that these compounds bind directly to Nef and may interfere with Nef dimerization, which we have observed to be essential for function (see progress on Aim 2). Finally, these novel, Nef-directed compounds potently inhibited the replication of all 10 chimeric forms of HIV-1 in both U87MG and CEM-T4 cells, suggesting that they recognize a shared structural feature of Nef (e.g., the dimer interface).

<u>Progress on Aim 2.</u> No progress made during this reporting period.

Research Project 2: Project Title and Purpose

Alterations in Bioenergetics and Mitochondrial Function in Tumor Cells - Cells generate the energy they need to function and divide through two different metabolic pathways, mitochondrial oxidative phosphorylation and glycolysis. Due to their rapid growth, tumor cells have unique energy demands. However, the mechanism by which tumor cells regulate energy production is poorly understood. This project will examine the balance between these two energy producing pathways in tumor cells. The cells to be examined are associated with specific genetic alterations in critical genes (oncogenes, like Myc and Ras) or include critical genes that have been mutated (tumor suppressor genes, like p53). This project also addresses how shifts in energy demands can increase genome instabilities like mutations. Understanding bioenergetic shifts of specific tumor types may improve therapeutic approaches.

Duration of Project

1/1/2009 - 12/31/2010

Project Overview

Tumor cells are rapidly dividing cells with unique metabolic demands for generating cellular constituents and energy. The purpose of this project is to understand how tumor cells adjust their bioenergetic demands through the regulation of glycolysis and oxidative phosphorylation as well as how mitochondrial dysfunction contributes to the cellular transformation process from a normal cell to a neoplastic cell. This project will address three specific aims. The first aim examines the balance between glycolysis and oxidative phosphorylation (OXPHOS), mitochondrial mutations, and the generation of reactive oxygen species (ROS) in a number of common tumor cell lines, some of which are included in the NCI 60 panel. Oxygen consumption during OXPHOS and production of lactate will be simultaneously measured using the Seahorse Bioscience XF24 flux analyzer. Mitochondrial DNA mutations will be detected by direct DNA sequencing, and mitochondrial and nuclear DNA damage will be assessed by a quantitative polymerase chain reaction (PCR) assay. ROS will be evaluated using fluorescent probes. The second aim defines the role of specific cancer-causing genes in affecting the energy balance. The specific oncogenes to be examined are: C-Myc, Ras, p53, and HMGB-1. The third aim examines whether the energy balance in cancer stem cells differs from that in general tumor cells. Stem cells from two breast cancer (MCF-7 and MDA-MB-231) lines will be investigated. In addition, energetic profiles of induced-pluripotent stem cells (expressing four genes: Oct4, Sox2, Klf4, and Myc) derived from human fibroblasts will be examined. It is hypothesized that stem cells will have the greatest capacity to adapt to specific energetic demands and will be more resistant to genotoxic agents like hydrogen peroxide.

Principal Investigator

Bennett Van Houten, PhD
Richard M. Cyert Professor of Molecular Oncology and Professor of Pharmacology and Chemical Biology; Director, Molecular and Cell Biology Program
University of Pittsburgh

Hillman Cancer Center
5117 Centre Avenue
Research Pavilion, Suite 2.6
Pittsburgh, PA 15213-1863

Other Participating Researchers

None

Expected Research Outcomes and Benefits

While there has been resurgence in the hypothesis that tumors have high glycolytic potential, careful, systematic analysis of the bioenergetic balance between oxidative phosphorylation and glycolysis in a wide range of tumor types is lacking. This project will ascertain the degree to which particular tumor types are dependent on a given bioenergetic pathway. More specifically, this project tests the hypothesis that deregulation of normal mitochondrial function constitutes a selective advantage to the evolution of a highly metastatic tumor. This altered mitochondrial function manifests itself through the production of increased ROS and greater genome instability in both the mitochondrial and nuclear genomes. Validation of these ideas through tissue culture models will allow the development of potential biomarkers to be used in future screening of primary tumors. Understanding the bioenergetic and metabolic demands of specific tumor types grown in culture will facilitate preclinical assays and the development of specific inhibitors of these pathways as potential chemotherapeutic agents. Thus, this project serves as a foundation for future xenograft tumor models in nude mice and specific mouse tumor models. Elucidation of the relative importance of particular metabolic pathways to the growth of specific tumor types will foster improved therapeutic approaches.

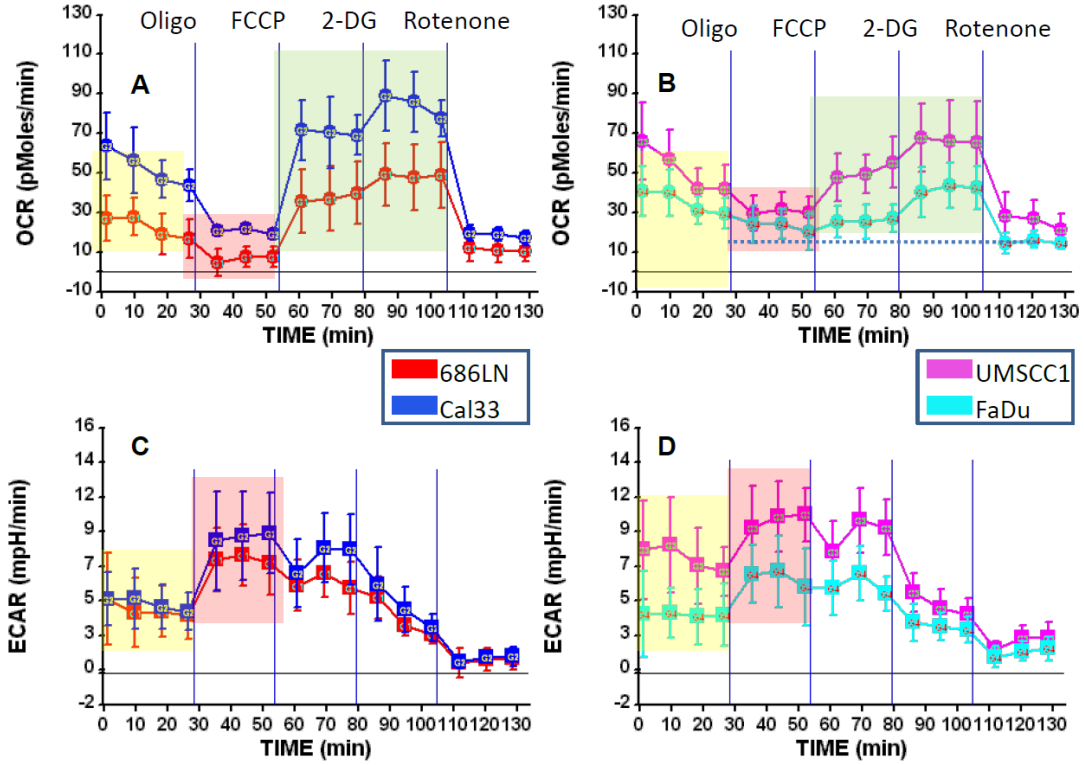
Summary of Research Completed

During this funding period, we continued to examine metabolic differences in various cancer cell types using a novel and innovative instrument for the analysis of tumor cell bioenergetics, the Seahorse XF24 Flux analyzer (Seahorse Biosciences, Billerica, MA). This instrument employs a dual fluorescent probe, which simultaneously measures two indices of energy production (OXPHOS and glycolysis), including the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR). The OCR directly quantifies OXPHOS, in which oxygen is consumed to form water through a four-electron reduction at complex IV of the electron transport chain. The major source of proton production is lactate made by glycolysis.

Using this instrument, in collaboration with Dr. Sufi Thomas at the University of Pittsburgh (who supplied the cell lines), we have begun to study four of 10 different head and neck squamous carcinoma (HNSCC) cell lines and found remarkable variability in their bioenergetic profiles. Figure 1 shows real time measurement of four different HNSCC cell lines (686LN, Cal33, FaDu, and UMSCC1) grown in 25 mM glucose/Dulbecco's Modified Eagle Medium (DMEM). Using four pharmacological inhibitors (oligomycin, carbonyl cyanide p-trifluoromethoxyphenylhydrazone/FCCP, 2-deoxyglucose, and rotenone), oxygen consumption can be directly linked to adenosine triphosphate (ATP) production, maximum respiration

capacity, and mitochondrial membrane leak. As depicted in this figure, the yellow regions show the basal oxygen consumption (panels A and B) and basal rates of glycolysis (panels C and D). Cal33 and UMSCC1 cells have a high basal rate of OXPHOS, and UMSCC1 cells have the highest basal rate of glycolysis (panel D). The regions in red show the effect of oligomycin, indicating how cells respond to inhibition of OXPHOS with a concomitant increase in glycolysis. The green region shows the reserve OXPHOS capacity. Note that Cal33 (highest), 686LN, and UMSCC1 all have appreciable reserve OXPHOS capacity, while FaDu does not. Finally, subtracting the values after rotenone addition from the rates after oligomycin (dotted line in panel B) reveals that the FaDu cell line shows uncoupled mitochondria with appreciable proton leak. These studies confirm that tumor cell lines have different intrinsic bioenergetic profiles. We are currently examining the underlying molecular basis of these differences.

Figure 1
Analysis of cellular bioenergentics in four HNSCC cell lines. 686LN, Cal33, UMSCC1, and FaDu were analyzed for OCR, a measure of OXPHOS, and extracellular acidification rate (ECAR), a measure of lactate production by glycolysis. These data represent the mean +/- standard deviation of five replicate wells with two biological repeats. Oligo, oligomycin, an inhibitor of complex V that blocks oxygen consumption; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazine, an uncoupler of proton flux and oxygen consumption that gives a measure of reserve OXPHOS capacity; 2-DG, 2-deoxyglucose, an inhibitor of glucose uptake and glycolysis; and rotenone, an inhibitor of complex I.



Research Project 3: Project Title and Purpose

Clinical Trials in Melanoma - The University of Pittsburgh Cancer Institute's (UPCI) Clinical Research Service (CRS) provides valuable resources for researchers and clinicians seeking to improve clinical research as a means to elevate patient care standards and treatment efficacy. This project will evaluate the clinical response of melanoma patients at various disease stages to novel therapeutic regimens designed to be specific to their treatment needs.

Duration of Project

1/1/2009 - 12/31/2010

Project Overview

E1697 (UPCI 99-006): This intergroup international adjuvant study aims to determine whether four weeks of high dose interferon (IFN)-α2b improves response rate compared to standard observation for patients with resected melanoma of intermediate to high risk of recurrence. Of 1,420 patients planned for study accrual, more than 800 have been accrued to date (57 registered at UPCI out of 80 screened). Blood samples will be banked, and corollary laboratory translational studies of prognostic and predictive biomarkers of disease outcome and therapeutic benefit will be performed. These analyses will enlarge upon careful studies of the biomarkers of tumor progression (prognostic biomarkers) and response to IFN therapy (predictive biomarkers).

E2602 (UPCI 03-111): This phase II study examines low-dose polyethylene glycol (PEG)-coupled IFN-α2b as a long-acting and potentially more effective anti-angiogenic agent in patients with metastatic melanoma and an elevated plasma level of basic fibroblast growth factor (b-FGF > 15 pg/mL). This trial directly tests the role of b-FGF as a promoter of angiogenesis in melanoma progression. UPCI has enrolled a preponderance of the patients (25 registered out of 42 screened) who have been enrolled into this study nationally, where the strictures for disease status, elevated b-FGF levels, and monitoring of b-FGF levels have been challenging.

UPCI 05-125: This phase II clinical and translational study tests the safety and efficacy of combination biotherapy with high-dose IFN-α2b and anti-cytotoxic T-lymphocyte antigen 4 (CTLA4) monoclonal antibody CP-675,206 for recurrent inoperable stage III or stage IV melanoma. Each agent has been individually shown to induce major objective durable responses in 5-10 percent of patients, and each is correlated with the induction of autoimmunity to host endocrine and cutaneous, as well as GI, target organs. We expect these agents to synergize to induce greater antitumor autoimmune responses, with augmented clinical antitumor efficacy. To date, UPCI has enrolled 23 of 30 patients screened, and the trial has passed first-phase efficacy thresholds for continued accrual. Accrual of 20 additional patients is planned.

UPCI 07-008: This phase I/II trial examines the combination of decitabine and temozolomide for treating patients with unresectable metastatic melanoma. This drug combination is designed to abrogate chemotherapy resistance based on the modulation of two deoxyribonucleic acid (DNA) repair pathways. Up to 18 patients may be accrued in phase I, and up to 60 patients may be accrued overall. To date, three patients of five screened have been registered.

Principal Investigator

John M. Kirkwood, MD
Professor of Medicine and Director, Melanoma Center
University of Pittsburgh
Hillman Cancer Research Pavilion
5117 Centre Avenue
Suite 1.32
Pittsburgh, PA 15213

Other Participating Researchers

None

Expected Research Outcomes and Benefits

E1697 (UPCI 99-006): At least 30 percent of all newly diagnosed stage IIA-B and IIIA melanoma patients will experience recurrence at five years, with subsequent mortality. An effective adjuvant therapy would, therefore, greatly benefit patients. This study evaluates postoperative therapy with high-dose IFN-α2b for melanoma patients at intermediate risk of recurrence and death after surgical resection. The planned corollary studies will contribute to our understanding of potential prognostic and predictive biomarkers to refine patient selection and improve the therapeutic index of adjuvant IFN therapy. This treatment regimen, which was developed at UPCI, remains the only demonstrably effective therapy for high-risk melanoma, with consistent and highly significant worldwide relapse-free survival benefits as well as individual trial overall survival benefits.

E2602 (UPCI 03-111): Growth factors like b-FGF drive melanoma progression and metastasis. This study will determine whether circulating b-FGF can be suppressed to normal levels when treated with a new and long-acting PEG-IFNα2b. This study represents one of the first efforts at targeted melanoma therapy, providing an innovative approach with the potential to improve patient survival.

UPCI 05-125: Autoimmunity is induced by high-dose IFNα therapy as well as with anti-CTLA4 blocking antibodies and is correlated with therapeutic benefit. Treatment with a combination of these two agents is expected to improve response rate and quality/durability of response in patients with recurrent inoperable stage III and stage IV melanoma. Our therapeutic target is to achieve a 20 percent or better rate of objective response, complete response, or partial response, compared to the 5 to 10 percent expected in study-eligible patients.

UPCI 07-008: This study will test the ability of decitabine to reverse drug resistance to the chemotherapy temozolomide in patients with advanced metastatic melanoma. This treatment regimen is expected to result in an overall improvement in response rate of metastatic melanoma from 7 to 21 percent.

Summary of Research Completed

E1697 (UPCI 09-006): A total of 105 participants have been screened and 64 have been enrolled at the University of Pittsburgh. Analysis has shown that one month of IFN treatment is not superior to observation, leading us to conclude that for high-risk melanoma patients, IFN therapy should be planned for one year. This important study has resolved an unanswered question regarding the role of one month of therapy and indicates that the current standard (one year) should remain in place.

E2602 (UPCI 03-111): The U.S. Food and Drug Administration (FDA) approved pegylated interferon as an adjuvant therapy for patients with high-risk for relapse melanoma in March 2011. Since January 1, 2010, 14 patients have been screened for the study; however, no patients have been accrued due to screen failures related to the strict criteria for high plasma b-FGF levels. Since March 2004, our center has accrued 15 patients on this trial.

UPCI 05-125: To date, UPCI has enrolled 37 patients, completing accrual for this trial. Efficacy thresholds have been passed for further development of this regimen, including promising tumor response rate, relapse-free survival rate, and overall survival rate. We are planning to submit this regimen for consideration as a national cooperative group Phase II randomized trial.

UPCI 07-008: This Phase I/II trial was completed in 2010, and follow-up for all patients enrolled is ongoing. A total of 39 patients were accrued for this study. The first 10 patients were accrued to the Phase I portion, which determined that a dose of decitabine of 0.15 mg/kg daily for 10 days was safely added to temozolomide given in extended schedule. The Phase II portion accrued an additional 29 patients. At the end of the study, 33 patients were treated on the Phase II recommended dose and considered evaluable for the efficacy analysis. Of those, 15 percent of patients had an objective response that met the primary endpoint of the study. An additional 32 percent had shrinkage of tumor or stable disease, for a total clinical benefit rate of 57 percent, exceeding the reported rates of 32 percent and 37 percent in large Phase III trials testing the same regimen of temozolomide alone. The median overall survival was 15.2 months, exceeding the expected survival of 7.9 months for this population. In addition, the one-year overall survival rate more than doubled from an expected 25 percent to an observed 63 percent. This study demonstrated that decitabine can be safely added to temozolomide in patients with metastatic melanoma at biologically relevant doses and seems to reverse the resistance of melanoma to chemotherapy, thereby significantly improving the efficacy of chemotherapy in patients with metastatic melanoma. We continue to actively follow all study patients for survival.

Research Project 4: Project Title and Purpose

Therapeutic Vaccine Trials in Recurrent Prostate Cancer - The University of Pittsburgh Cancer Institute's (UPCI) Clinical Research Service (CRS) provides valuable resources for researchers and clinicians seeking to improve clinical research to elevate patient care standards and treatment efficacy. This project will conduct two novel vaccine trials in patients with recurrent prostate cancer: (1) a safety and efficacy study of a new vaccine targeted against mucin-1 (MUC-1) in conjunction with poly-ICLC (polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose) in patients with recurrent and/or advanced prostate cancer and (2) a

safety and efficacy evaluation of alpha-type-1 dendritic cell (DC)-based vaccines loaded with allogeneic prostate tumor cells in combination with androgen ablation in patients with recurrent prostate cancer.

Duration of Project

1/1/2009 - 12/31/2010

Project Overview

MUC-1 and poly-ICLC trial
This clinical trial will test a MUC-1 vaccine in conjunction with poly-ICLC in patients with recurrent and/or advanced prostate cancer. The primary objective will be to evaluate the efficacy of poly-ICLC in boosting the immunologic response of a MUC-1 peptide vaccine. The definition of an immunologic response is based on observing an increase from pre- to post-vaccine in the count of peripheral blood CD8+ T cells that react to the MUC-1 peptide in an enzyme-linked immunosorbent spot (ELISPOT) assay. A total of 14 patients will be accrued to this trial.

Evaluation of αDC-1 vaccine in combination with androgen ablation
This trial will evaluate the feasibility, safety, and efficacy of intradermal vaccination of prostate cancer patients with alpha-type-1-polarized dendritic cells (αDC-1) loaded with apoptotic allogeneic tumor cells (LNCap). The study will target men with recurrent prostate cancer who have failed local therapy and have no measurable metastasis but have a rising prostate-specific antigen (PSA) level with a doubling time of less than 10 months. In this two-arm study, patients will receive the vaccine in combination with limited androgen ablation (AA) and a luteinizing hormone-releasing hormone (LHRH) analog for three months. Patients will be randomly assigned to one of two cohorts. In cohort A, patients will first be treated with limited AA only for three months, and at the time of prostate-specific antigen (PSA) relapse (PSA $\geq$ 1 ng/dl), will receive the DC vaccine in conjunction with AA. In cohort B, the sequence of treatment will be reversed. The primary efficacy objective is to evaluate the effect of the αDC-1 vaccine on time to PSA progression compared to AA alone. PSA progression is defined as a rise in the PSA value to $\geq$ 1 ng/dl. A total of 12 to16 patients will be accrued in both arms of this study.

Principal Investigator

Gurkamal S. Chatta, MD
Associate Professor of Medicine
University of Pittsburgh
UPMC Cancer Pavilion
5150 Centre Avenue, Suite 564
Pittsburgh, PA 15232

Other Participating Researchers

None

Expected Research Outcomes and Benefits

MUC-1 and poly-ICLC trial

This clinical study is expected to result in the development of a safe and effective vaccine treatment regimen for men with advanced prostate cancer. Standard treatment for advanced prostate cancer (androgen ablation and chemotherapy) is limited, primarily palliative, and associated with significant side effects. Prostate-specific vaccine therapy, if effective, offers the prospect of prolonging survival with minimal side effects.

Evaluation of αDC-1 vaccine in combination with androgen ablation

In this study, we aim to develop a novel vaccine to effectively treat men with prostate cancer that has recurred following failed local therapy (surgery and/or radiation) and that is detectable only by a rise in serum PSA levels (PSA-only disease). This patient population is ideal for an immunologic intervention given that: (1) this group is the largest subpopulation of men with prostate cancer in the U.S., (2) there is no standard treatment for this subgroup, and (3) this subgroup has minimal systemic disease burden. We will carefully monitor immunological responses to vaccination at different time points, but primary indications of vaccine efficacy will be stabilized patient PSA levels and prolonged time between treatment and disease relapse (PSA progression).

Summary of Research Completed

MUC-1 and poly-ICLC trial

Results from the Phase I portion of this trial were recently presented at the American Society of Clinical Oncology (ASCO) meeting in Chicago and are summarized below. Based on these results, the Phase 2 portion of the protocol has been modified and is currently being reviewed by the University of Pittsburgh Institutional Review Board.

A single-arm study evaluated the *in vivo* efficacy of poly-ICLC at a dose of 25 μg/kg. Eligible patients were men with advanced prostate cancer (PCa) and evidence of systemic immunosuppression (defined as less than 30 percent of peripheral T-cells expressing interferon gamma [IFN-γ]).

Fourteen patients were recruited for the study, with ages ranging from 51 to 80 years (median 72). Thirteen participants had castrate-resistant disease, and 12 had evidence of metastatic disease. In weeks 1 and 2 of the study, participants were pretreated with intramuscular (i.m.) injections of poly-ICLC (25μg/kg) three days per week. In weeks 3, 5, and 7, participants were treated with subcutaneous injections of MUC-1 vaccine (100μg). A dose of tetanus toxoid was administered via i.m. injection in week 3. Granulocyte macrophage colony stimulating factor (GM-CSF) (100μg) was administered on days 2-4 of the weeks of MUC-1 injection. Participants continued to receive poly-ICLC twice weekly while they remained in the study (Figure 1).

Cytokine levels were measured by Luminex assay prior to pretreatment and again at week 3, and by ELISPOT prior to pretreatment and again at week 5. Nine of 14 participants demonstrated increased IFN-γ production ranging from 15 to 76 percent. Twelve of 14 participants showed an

increase in tumor necrosis factor (TNF)-α production that ranged from 15 to 137 percent. All participants who showed an increase in IFN-γ also showed increases in TNF-α (Figure 2).

Evaluation of T-cell subsets by ELISPOT showed two participants who had increased expression of IFN-γ in $CD4^+$ T-cells; five participants demonstrated increased expression of IFN-γ in $CD8^+$ T-cells. Upregulation of TNF-α expression was observed in both $CD4^+$ and $CD8^+$ T-cells in eight of 14 participants (Figure 3).

DC phenotype was evaluated by flow cytometry at baseline and at weeks 3 and 9. Upregulation of CD80 and CD86 was observed only in those participants with low levels of expression at baseline.

Anti-MUC-1 antibody levels were measured at baseline by enzyme-linked immunosorbent assay (ELISA) and followed on weeks 3, 5, 7, and 9. Four participants showed at least a 25 percent percent increase in both anti-MUC-1 IgM and IgG levels between weeks 3 and 9 (Figures 4 and 5). Of these, three participants demonstrated increases in both TNF-α and IFN-γ production when measured by Luminex assay after poly-ICLC pretreatment.

Serum tetanus antitoxin levels were measured at weeks 3 and 9. Only one participant showed low anti-tetanus antibody titers postimmunization, consistent with anergy. This participant had no upregulation of either cytokine production or anti-MUC-1 antibody titers.

Disease progression was defined according to the outcome measures recommended by the Prostate Cancer Clinical Trials Working Group 2 (PCWG2). PSA values were measured serially while participants remained on the study protocol (Figure 6). Imaging studies were performed as clinically appropriate. Two participants remained without evidence of disease progression for over 20 weeks. Both of these participants demonstrated increased levels of IFN-γ and TNF-α after pretreatment with poly-ICLC. Five participants demonstrated evidence of disease progression on either bone scan or computed tomography (CT) scan. One participant was removed from the protocol after hospitalization with acute kidney injury, though the injury was believed to be unrelated to the study interventions.

In conclusion, poly-ICLC at a dose of 25μg/kg reversed systemic immunosuppression in nine of 14 participants with advanced PCa, as demonstrated by increased levels of IFN-γ and TNF-α. Coupled with the encouraging immune and clinical responses observed in some patients, this finding warrants further investigation of poly-ICLC as a vaccine adjuvant in advanced PCa.

<u>Evaluation of αDC-1 vaccine in combination with androgen ablation</u>
To date, nine of 16 patients have been accrued to this study. We anticipate completing accrual by late 2012.

Figure 1. Study Schema.

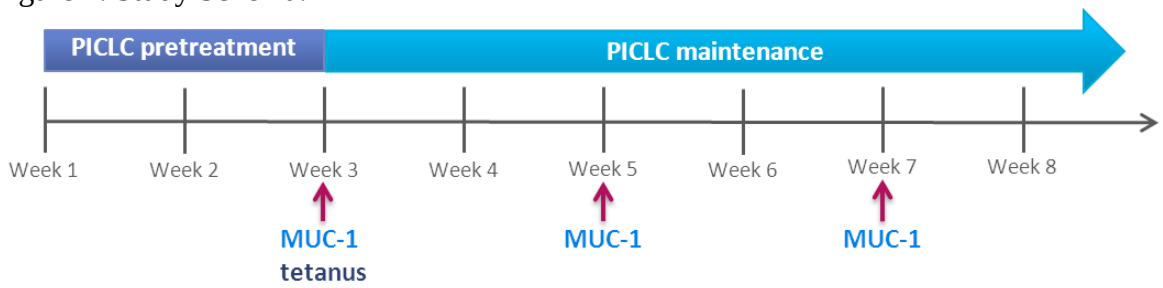
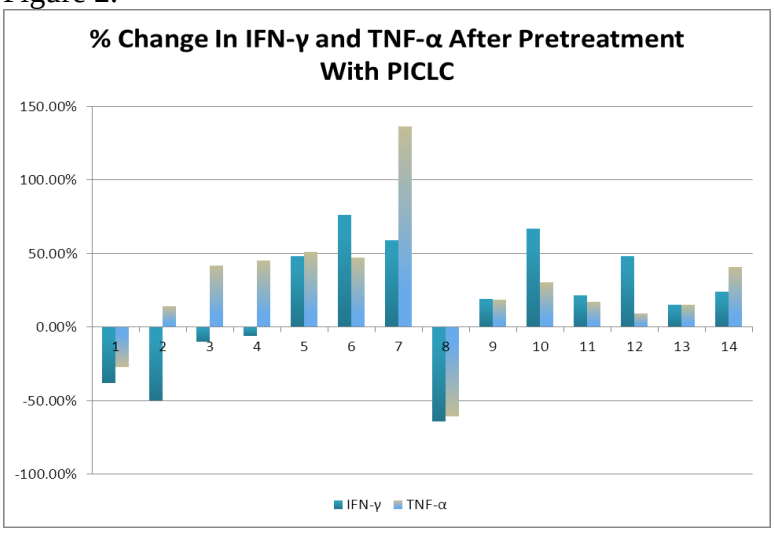


Figure 2.



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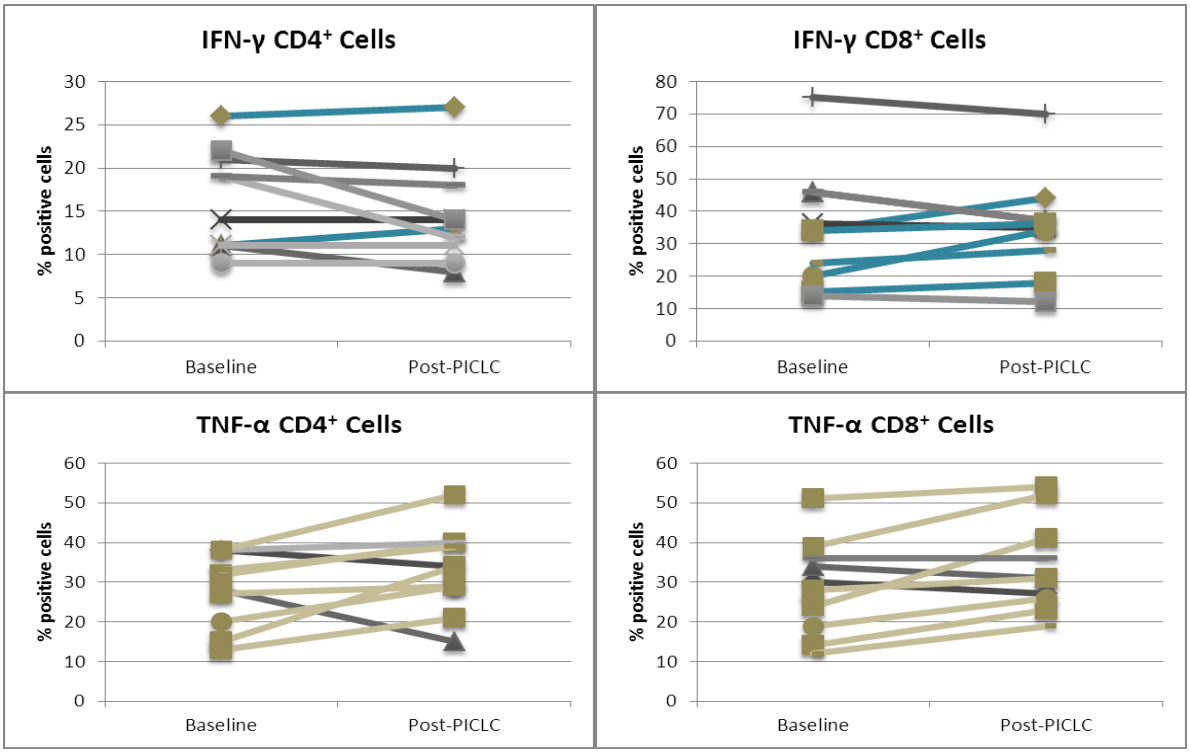


Figure 4.

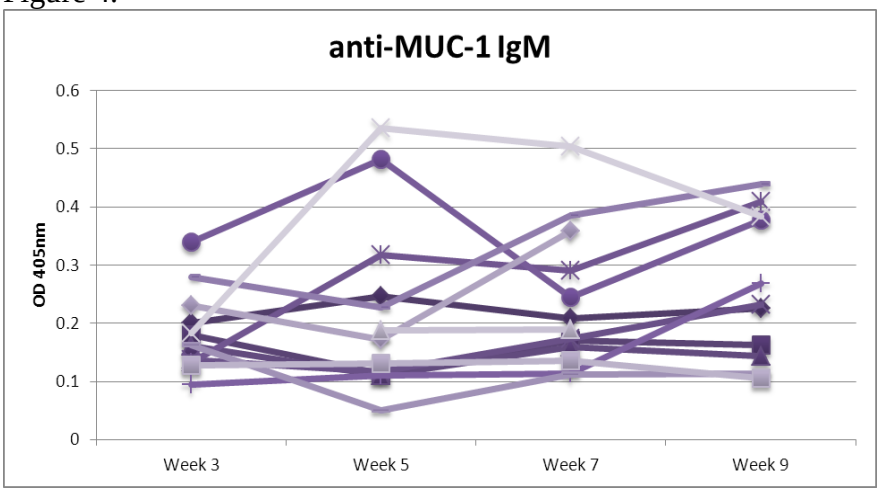


Figure 5.

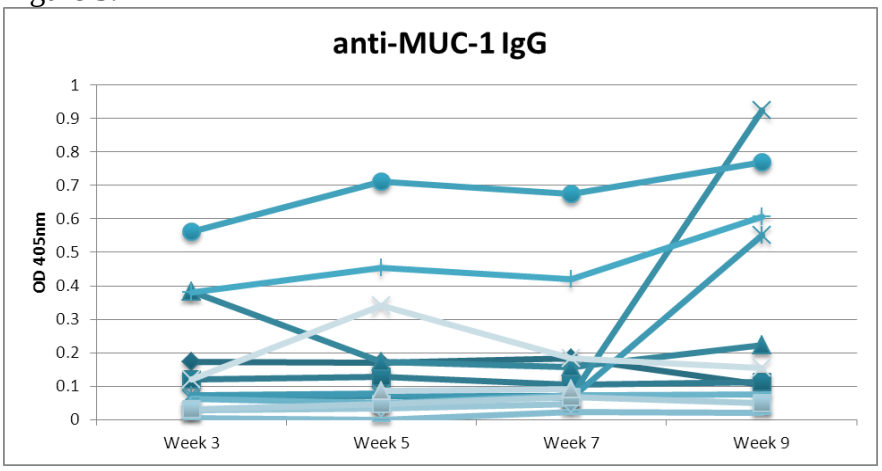
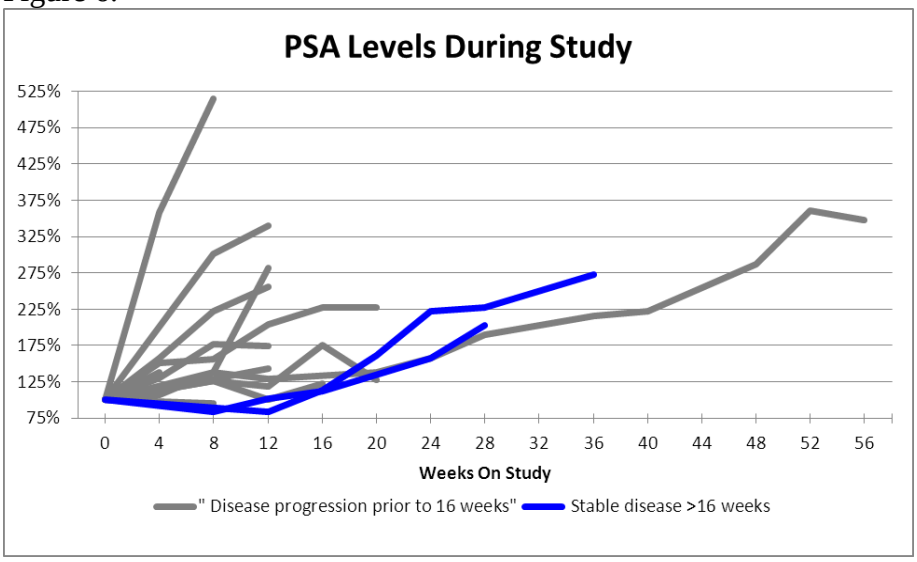


Figure 6.



Research Project 5: Project Title and Purpose

Research Infrastructure: Bridgeside Point II Building Renovations - Bridgeside Point II (BPII), the University of Pittsburgh's newest research facility, is nearing completion. This project will address final construction and laboratory fit-out in the building. By establishing this additional space, the Department of Orthopaedic Surgery, the Department of Microbiology and Molecular Genetics, and the McGowan Institute for Regenerative Medicine will be able to expand their research programs, recruit additional faculty members, and accommodate researchers' changing needs.

Duration of Project

12/17/2009 – 9/30/2010

Project Overview

The aim of this project is to finalize construction and laboratory fit-out at Bridgeside Point II (BPII), the University of Pittsburgh's newest research facility. Existing laboratory space is limited, and new space is needed to keep pace with the needs of the University's biomedical research community. This project is designed to complete final construction at BPII, outfit the laboratory space with fume hoods and a data telecom system, install a sterilizer unit in the vivarium facility, and install a security system for the entire building.

Principal Investigator

Arthur S. Levine, MD
University of Pittsburgh
Suite 401 Scaife Hall
3550 Terrace Street
Pittsburgh, PA 15261

Other Participating Researchers

None

Expected Research Outcomes and Benefits

This project will enhance research infrastructure at the University of Pittsburgh by providing the additional research space required to expand research programs, recruit new faculty members, and accommodate the changing needs of existing investigations, all of which are essential to advance biomedical progress and, in the long run, to translate findings from animal to human studies.

Summary of Research Completed

The Department of Orthopaedic Surgery, the McGowan Institute for Regenerative Medicine, and the Department of Microbiology and Molecular Genetics have begun to use the new space full-time during the reporting period and continue to recruit new faculty to their respective departments. Lab space was also renovated to accommodate the Department of Psychiatry and Division of Laboratory Animal Resources (DLAR), including animal housing facilities and fume hoods. This renovation phase completes the specific aims of this project

Research Project 6: Project Title and Purpose

Estrogen Receptor Genetics in Breast Cancer - Breast cancer remains a major health issue in the United States, with approximately 210,000 new cases and 40,000 deaths per year. Breast cancer can be subdivided into estrogen receptor (ER) positive and negative disease. Breast cancers that express ER are often less aggressive and respond to anti-hormone therapy; however, a significant fraction of patients with ER positive disease do not respond to therapy. New sequencing technologies have recently allowed an unprecedented view of ER action in breast cancer. This project will investigate how genetic alterations in ER and the ER pathway affect response to the hormone and whether the ER itself is involved in generating genetic changes in breast cancer. Better knowledge of ER action should translate into improved treatment of ER positive breast cancer.

Duration of Project

3/30/2011 – 12/31/2012

Project Overview

ER is a major determinant of breast cancer initiation, progression, and response to anti-hormone therapy. Recent advances in molecular techniques have allowed an unprecedented view into how this hormone receptor orchestrates its biological response in normal cells and tumors. This project is based on the use of novel next-generation sequencing techniques, which have provided a unique insight into genetic changes in ER in human breast cancers and new ideas about how ER may effect genetic changes in breast cancer. This project will use an array of new technologies to comprehensively interrogate ER in breast cancer cell lines. In the first aim, we will study the effect of germline single nucleotide polymorphisms (SNPs) and cancer-specific somatic mutations on ER function. Preliminary studies using next-generation sequencing in human breast tumors have identified numerous genetic changes in ER, and we will examine these changes with specific regard to their effects on estrogen-mediated cancer phenotypes, such as proliferation and invasion. In the second aim, we will examine how alterations in the deoxyribonucleic acid (DNA) sequence that ER binds might affect subsequent hormone response. Target sites will be identified by chromatin immunoprecipitation and sequencing; and changes will be characterized, especially related to hormone response. In the third aim, we will examine whether ER binding sites are associated with regions of DNA translocations in breast cancer cells and whether ER can itself direct genomic translocation in cells with reduced DNA repair activity. This project will provide significant new information regarding ER action in

breast cancer and will likely have a major impact in improving prediction and response to hormone therapy.

Principal Investigator

Adrian V. Lee, PhD
University of Pittsburgh
Magee-Womens Research Institute
204 Craft Avenue
Room B705
Pittsburgh, PA 15213

Other Participating Researchers

Steffi Oesterreich, PhD - employed by University of Pittsburgh

Expected Research Outcomes and Benefits

Two-thirds (~130,000) of breast cancers detected in the United States each year are ER-positive and are treated with anti-hormone therapy. However, not all tumors respond to this therapy, and a majority will eventually develop resistance. This project will investigate genetic changes in ER and their role in causing differential responses to anti-hormone therapy. In Aim 1, we expect to find that somatic mutations in ER render the tumor resistant to anti-hormone therapy, indicating the need for alternative therapies. Similarly, germline polymorphisms in ER are likely to modulate the response to anti-hormone therapy; and some may actually render tumors hypersensitive to therapy, in which case the tumors may require anti-hormone therapy alone, thereby sparing the patient unnecessary toxic therapy. In Aim 2, we predict that genetic changes in ER binding sites will correlate with sensitivity or resistance to hormone therapy. Finally, we expect Aim 3 to produce novel data regarding how ER affects genomic translocations. The discovery of specific ER-directed genomic translocations will have major implications in breast cancer, as they may produce novel gene products that can act as tumor-specific diagnostic and therapeutic targets (similar to the oncogene fusion BCR-Abl in chronic myelogenous leukemia). In summary, this project will provide novel and critical information regarding ER function in breast cancer and is likely to have a major impact on hormone treatment of breast cancers.

Summary of Research Completed

This project has only been active for a short time, during which we have mainly focused on Aim 1, examining the role of genetic changes in the estrogen receptor (ESR1). We have also made some progress in Aim 2, in which we proposed to examine genome-wide binding of ER in breast cancer cell lines and to identify genetic changes in ER binding sites that affect hormone response.

Specific Aim 1: In a large next-generation sequencing approach using breast tumor DNA, we identified a large number of mutations and SNPs in ESR1. This finding is in stark contrast to a number of previous studies that failed to identify mutations in ESR1. This discrepancy could be

due to the high sensitivity of the deep sequencing methods or, alternatively, might represent an artifact of the more recently established high throughput technique and analysis approaches. Our goal at this time is to validate the findings from the next generation sequencing approach.

We identified a total of 60 putative mutations and six SNPs. This number decreases significantly when various stringency criteria are applied during the sequence analysis. We used very relaxed criteria in order to include all potential changes; therefore, the likelihood of false positives is increased. We are validating these changes using an orthogonal sequencing method from Sequenom, Inc., which is based on mass spectrometry analysis of DNA sequence.

Interestingly, the changes we identified were clustered in specific functional regions, such as the DNA binding domain and the activation domain 2 (AD2), which also harbors the ligand binding domain. In collaboration with the National Surgical Adjuvant Breast and Bowel Project (NSABP) in Pittsburgh, we have designed primers to confirm the genetic changes we originally identified in the breast tumors and will attempt to confirm the changes using the Sequenom methodology. We have designed 62 assays (for a total of 62 genetic changes, including some positive controls) fitting into nine reactions. We will first test the assays using DNA from a number of cell lines and will subsequently confirm the changes in the tumor samples.

Specific Aim 2: We have made progress in establishing the methods and the informatics analysis of a coupled chromatin immunoprecipitation sequencing (ChIP-seq) and gene expression array technique. We have successfully performed the studies in ER-transfected U2OS osteosarcoma cells and have initiated a collaboration with Dr. Xinghua Lu's group in the University of Pittsburgh's Department of Biomedical Informatics, who is currently performing informatics analyses. This experimental approach is rather complex, but our preliminary data suggest that the wet-bench experiments and the subsequent analyses are functional. Briefly, we have identified 61,127 ER binding sites in the presence of estrogen, the majority of which can be found in enhancer regions (within 50 kb of the transcriptional start sites). The majority of estrogen-regulated genes (509/721=71 percent) have ER biding sites; however, most ER binding sites are non-functional. Others have previously described this expected finding. We are currently trying to identify another factor that might be involved in rendering non-functional binding sites into active ones and are setting up similar assays in breast cancer cells.