University of Pittsburgh

Annual Progress Report: 2010 Formula Grant

Reporting Period

July 1, 2011-June 30, 2012

Formula Grant Overview

The University of Pittsburgh received $8,236,620 in formula funds for the grant award period January 1, 2011 through December 31, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

*Cellular Systems Biology in Cancer Drug Discovery* - The project focuses on the development of a research program to discover and develop small molecule anticancer therapeutics. At the University of Pittsburgh, novel chemistries are combined with cellular systems biology and computational pharmacology approaches to drug discovery. The overriding goal of this project is to further enhance the broad scientific capabilities at the University of Pittsburgh in these research areas and to deploy them for the development of novel anticancer drug candidates and, ultimately, commercial drugs.

Anticipated Duration of Project

1/1/2011 - 12/31/2014

Project Overview

The pharmaceutical industry has been hampered in developing new drugs, including cancer drugs, by the complexity of biological systems that give rise to on- and off-target effects of lead compounds in living systems. It is now understood that life is based on integrated, interacting networks of genes, proteins, and metabolic processes that give rise to either normal or, in disease, abnormal functions. This complex “systems biology” must be addressed to create a safe and effective drug. The pharmaceutical industry has, instead, focused primarily on simplifying biological testing to speed up the process. The result has been a serious failure rate (>90%) in getting drug candidates safely through the drug discovery and development process. However, academic groups are ideally positioned to investigate cancer biology and potential off-target compound toxicity by directly studying this complexity with more sophisticated cell-based model systems and computational methods. The University of Pittsburgh Drug Discovery Institute (UPDDI) has been integrating its expertise in cancer biology, chemistry, medicinal chemistry, safety assessment, and computational molecular and systems biology to pursue novel cancer drug discovery. The goal of the present project is to advance anticancer drug discovery by integrating current activities with novel chemistry, systems biology, and computational
pharmacology approaches and applying the new methodology to developing three classes of targets: (1) improved modulators of microtubule dynamics, (2) transcription factors (e.g., STAT3 [signal transducer and activator of transcription]), and (3) modulators of the interaction between two critical proteins (e.g., Myc-Max) in an important cellular signaling pathway (Myc is also a transcription factor). Functional, cell-based models using human cell lines, primary cells, and ultimately validated stem cell-derived cells will be developed and investigated using medium-throughput and high-content light microscope imaging methods, as well as mechanistic probe molecules, to define the systemic response to both commercially available and novel drug candidates. Computational methods will be developed and applied to assess the protein-protein and protein-inhibitor interactions at the molecular structural level and to relate the cellular responses to known patterns of gene and protein responses, cellular pathways, and protein-protein interaction networks. Critical attention will be given to polypharmacology effects for early safety assessment and compound prioritization. Promising compounds will be further developed by lead optimization and pharmacokinetics studies, including animal models.

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**Other Participating Researchers**

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**Expected Research Outcomes and Benefits**

This project will harness collaborations across the University of Pittsburgh from multiple departments and institutes to create novel compounds that can be progressed toward effective therapeutics. A key outcome will be to progress one or more compounds to the point at which there is significant interest by the pharmaceutical industry to either license the candidate drug or collaborate with UPDDI to develop the candidate further. The project will increase the University’s research capacity by creating new opportunities in tissue engineering, chemical synthesis, systems biology, and computational biology and should yield new technologies that can be licensed to industry partners. The University of Pittsburgh has a strong presence in cancer biology with the University of Pittsburgh Cancer Institute (UPCI) and has significant expertise and federal funding that can facilitate the drug discovery process. Together with the Departments of Pharmaceutical Sciences, Chemistry, and Computational and Systems Biology, UPDDI is also a member of the Chemical Biology Consortium (CBC) of the National Cancer Institute, and the Department of Chemistry hosts one of five National Institutes of General Medical Sciences (NIGMS)-sponsored Centers for Chemical Methodologies and Library Development. These centers provide the researchers access to chemical libraries and broad collaborations with other
academic centers of excellence working on cancer therapies. The application of a cellular systems biology approach increases the potential of a more personalized medicine outcome for sub-populations when stem cells replace the human cell lines and primary cells in the future.

Summary of Research Completed

We have made progress toward our specific aims and met several milestones proposed for the first reporting period of this project.

Milestones for the period 7/1/2011-6/30/2012

1. Implement the rat hepatocyte and HepG2 early safety assessment, and Cellular Systems Biology (CSB™) panels and publish the results

   Progress: Assays for early safety assessment were developed using zebrafish embryos, rat primary hepatocytes, and human hepatocellular carcinoma (HepG2) cells. Traditionally, it has been difficult to assess cardiotoxicity in vitro, using the standard cell-based models. We are developing a zebrafish embryo model, as a convenient vertebrate organism, to screen for cardiotoxicity (Figure 1). The primary rat hepatocyte and human hepatocyte cell line models were developed specifically to identify clinically relevant toxic mechanisms of action. The subcellular component microsome assay was implemented to screen compounds for clearance and drug metabolism issues. Taken together, these assay profiles have been used to identify safety issues for two major medicinal chemistry programs within UPDDI (Figure 2) and will be used for this project. A manuscript is in preparation.

2. Develop a lead series for MT stabilizers and STAT3 and Myc-Max inhibitors.

   Progress: A major aim of this project is to characterize the heterogeneity of phenotypic and molecular responses to compound stimuli in populations of cancer cells, including microtubule (MT) stabilizers, STAT3 transcription factor inhibitors, and Myc-Max gene inhibitors. As a result of our preliminary investigations into the heterogeneity of responses to stimulation with interferon gamma (IFN-γ) and interleukin 6 (IL-6) in the head and neck cancer cell line Cal33 and a reference cancer cell line, HeLa, we have identified a need for imaging standards and a method for system calibration to achieve accurate quantitation of the cellular phenotypes proposed for this study. To address this need, we have adapted robust fluorescent-bead standards used in flow cytometry and used parallel studies by flow cytometry to validate the calibration methods we have applied to imaging. We now have the protocols in place to allow quantitative profiling of heterogeneity in response to stimulation with compound series.

3. Develop additional funding to pursue preclinical testing.

   Progress: Our strategy for preclinical testing is to develop and validate sophisticated 3D human organ and cancer models. To fund this development, we submitted an application to the National Institutes of Health (NIH) program—RFA-RM-11-022—Integrated Microphysiological Systems for Drug Efficacy and Toxicity Testing in Human Health and Disease ($8,661,139 in total direct costs). The goal of this project is to construct a microfluidic liver module that mimics the functions and responses of the human liver, with readouts designed to indicate both normal function and toxic responses. This module will be designed to integrate with other organ models to form a human microphysiology platform that will improve drug efficacy and safety testing. Our application received a favorable review, and we anticipate receiving a notice of award soon. Additional applications are currently in development.
4. **Implement computational tools to evaluate the Computational and Systems Biology (CSB) secondary screen profiling and early safety assessment profiling to optimally prioritize and/or de-risk leads and publish the results**

**Progress:** Our approach to secondary screening and early safety assessment will be focused on evaluating cellular responses in all subpopulations of cells. Subpopulations of cells often exhibit a different response than the population average; as a result, for example, they can survive toxic challenge. To extract information on subpopulation responses, we have implemented ModFit, a program developed to analyze cell cycle distributions in flow cytometry data that employs a Gaussian mixture model (GMM) approach to segmenting multiparameter phenotypic distributions. Based on the success of this method, we are generalizing the GMM modeling.

**Methods**

**Early safety assessment:** The transparency of the zebrafish embryo allows the capture of the beating heart by simple microscopic videography at 13 frames per second. We have developed novel image processing algorithms to calculate heart rate, beat periodicity, and contractile strength (force of contraction). Motion of the heart in phase contrast images (Figure 1A) is calculated from frame-to-frame pixel intensity differences (Figures 1B and 1C), which are analyzed by fast Fourier transform (FFT) algorithm to give a beat frequency. Further analysis by a series of short-term FFT measures the variability in the beat frequency, known as the rhythmicity index, which is characterized by the coefficient of variation (CV) of the beat frequency. The assay for beat rate was validated using compounds that are known to alter the beat rate in humans, such as terfenadine (Figure 1C).

For early safety profiling in cells, cultures of HepG2 cells from ATCC (American Type Culture Collection) and freshly isolated rat hepatocytes were plated in 384-well microplates and maintained at 37 degrees Celsius and 5 percent carbon dioxide. HepG2 cells were exposed to compounds for periods of one and 72 hours, stained with tetramethylrhodamine, ethyl ester, perchlorate (TMRE) and Hoechst 33342, then scanned on the ArrayScan VTI (Thermo-Fisher) and analyzed for mitochondrial potential and cell loss. Similarly, rat hepatocytes were exposed to compounds for one hour and labeled with TMRE and Hoechst 33342 for mitochondrial potential analysis, or four hours and labeled with dihydroethidium for reactive oxygen species (ROS) analysis on the ArrayScan VTI. Cytotoxicity was measured after five-day exposure of rat hepatocytes in overlay culture by labeling dead cells with propidium iodide and all cells with Hoechst 33342. All cell-based measures were done in duplicate 10-point, dose-response format, and inhibitor concentration reductions by half (IC50s) were calculated in Prism (GraphPad).

**Flow cytometric characterization of fluorescent bead standards:** To standardize and optimize our high content imaging systems, several sources of bead standards were obtained and run on a Becton Dickinson LSR II flow cytometer equipped with 355 nm, 404 nm, 488 nm, and 633 nm lasers. Data were collected on multiple channels simultaneously; the data were analyzed using FlowJo and/or ModFit to automatically determine the first peak CV and the ratio of the [second peak mean] / [first peak mean].

**Imaging system calibration and characterization:** The illumination and detection optics of imaging systems exhibit some fall-off in response across the field, often referred to as shading.
To compensate for this, we have implemented a flat-field correction protocol, for which we use a solution of fluorescent dye in a microplate as a uniform reference target for each fluorescent channel. The correction image is normalized to a mean of 1, and each image is divided by the reference to “flatten” the system response.

**Cell cycle measurements by flow cytometry and imaging:** To directly compare the DNA histograms of data acquired by flow and imaging cytometry, cultures grown in T 150 flasks were trypsinized, fixed with formaldehyde, permeabilized with Triton X-100, and stained with 2.5 μg/ml Hoechst 33342. The samples were divided in two; one was placed in suspension to be run on the LSR II flow cytometer, while the second was spun down at 1,500 g in 384-well plates to be run on the ArrayScan VTI high-content imager. We determined that media collected prior to trypsinization must be included in the sample to produce a full deoxyribonucleic acid (DNA) histogram. Data were analyzed using FlowJo and/or ModFit to automatically find the first peak in the intensity histogram, the CV, and the ratio of the [second peak mean] / [first peak mean].

**Computational methods for subpopulation analysis:** FlowJo and ModFit software were implemented for automated extraction of the cell-cycle compartments G0-G1, S, and G2-M. ModFit has the advantage of iteratively removing debris and aggregates from the histogram to yield a more accurate quantitation of compartments. ModFit performs a non-linear least squares fit to the data. Generalized multidimensional GMM is being implemented in R.

**Results**

**Early safety assessment.** Figure 1A illustrates the performance of the zebrafish cardiotoxicity assay. Using a set of control compounds, we have successfully shown that the beat-rate alteration in the zebrafish model parallels known effects of drugs on human heartbeat rates. Figure 1B shows the uniformity and amplitude of the measured beats in a control zebrafish embryo with a beat rate of 2.2 Hz; Figure 1C show the decrease in beat rate to 0.9 Hz and arrhythmia resulting from exposure to terfenadine. Figure 2 shows results from the application of the early safety profiling panels to a test set of compounds. This test set of 34 compounds, with a range of toxicity mechanisms of action (MOAs), shows the applicability of this panel to prioritization of compounds in a series, based on overall and specific MOAs.

**Flow cytometric characterization of bead standards for imaging.** The optimal DNA histogram analyzed by flow cytometry has tight CVs (<10 percent) in the G0/G1 and G2M peaks and an expected G2/G1 ratio of 2.0 the doubling of DNA prior to mitosis (Figure 3A). A survey of commercially available flow cytometric bead standards was performed by flow cytometry to quantify the resolution of our imaging system. The minimum CV of approximately 1.5 to 3.2 percent was observed in the DNA channels, while a range of CVs of 1.5 to 2.2 percent was observed in all other channels (data not shown). Additionally, intensities of 2-4 bead aggregates were used to confirm linearity of the channel of interest (Figure 4A and 4B).

**Flow cytometric validation of quantitative cell cycle analysis by imaging.** Comparisons of fixed HeLa and Cal33 tumor cell lines (Figure 5) shows that the subpopulations measured in the corrected images is within 1-2 percent of that extracted from the flow cytometry data (compare Figure 5A and 5B). The G2/G1 ratio of 2.0 demonstrates that the correction has not affected the linearity of the measurement. Figure 5C shows the imaging histogram prior to corrections,
demonstrating that the corrections greatly improved the quantitation of DNA content and the accurate segmentation of the subpopulations.

**Computational methods for subpopulation analysis.** ModFit analysis makes use of multiparameter measurements to segment overlapping cell cycle distributions between G0/G1, S, and G2/M, with rejection of debris, and gives consistent results in both flow cytometry and imaging, but is specifically designed for cell cycle analysis. We have implemented a package in R (http://www.R-project.org/) that makes use of higher order statistics to improve the sensitivity to subpopulations and a generalized multidimensional GMM model (Figure 6). These models will be used to relate subpopulation phenotypes to variations in pathway signaling.
Figure 1. Cardiotoxicity assessment in zebrafish embryos. (A) Phase contrast video sequences are analyzed for beat rate, rhythmicity, and force of contraction. (B) The control heart exhibits a very constant beat rate of 2.2 Hz, with a CV=0.09 percent. (C) Exposure to terfenadine, known to reduce heart rate, lowers the beat rate to about 0.4 Hz and induces some arrhythmia, with a CV=1.
Figure 2. Early Safety Profile. Example safety profile of 34 compounds tested in zebrafish, HepG2 cells, and primary rat hepatocytes. Although some compounds show broad toxicity effects (e.g. PCI1-04,06) others have more specific effects, such as mitochondrial function (e.g., PCI1-55) or moderate cardiotoxicity (e.g., JZ 1-78), illustrating the value of profiling for early safety assessment.
Figure 3. (A) The theoretical mammalian cell DNA histogram exhibits unitary peaks at G0/G1 and G2/M, S-phase heterogeneity, as well as variability in all compartments. (B) Actual DNA histogram from imaging cytometry with fit to G0/G2 peaks and S-phase using ModFit software. Extraction of cell cycle compartments from actual DNA histograms by ModFit depends on achieving CVs less than 10 percent and preferably less than 2 percent.

Figure 4. Comparison of the CV of the first peak in the 2µm fluorescence bead standard histograms from: (A) the LSR II flow cytometer and (B) the ArrayScan VTI imager. The excellent agreement of the imager intensity distributions with the bead specifications indicates the dramatic improvement in the resolution of single intensity peaks.
Figure 5. The effect of flat field correction on the extraction of cell cycle compartments by ModFit on imaging data. Cell cycle data were acquired on trypsinized/fixed cell cultures and stained with HO33342. (A) The standardized flow cytometric analysis is shown for comparison. (B) For image data collected on the ArrayScan VTI with flat field correction, the G0 peak in the histogram has a CV similar to the flow cytometry data, as well as a comparable percentage S. (C) The CVs of the G0 peak in the uncorrected (without flat field) images are extremely broadened, and the software was unable to find a significant G2M peak.

Figure 6. Statistical network models indicate mechanistic variability. (A) Higher-order statistics reveal finer details of cellular populations and facilitate the segmentation into subpopulations based on variation in functional response. (B) Higher-order statistics applied to multidimensional cellular data enable the characterization of local network topology variations in subpopulations.
Research Project 2: Project Title and Purpose

Complex Genetics of Congenital Heart Disease - This study will examine the complex genetics of congenital heart disease (CHD), specifically, the role of genetic variants of genes encoding ciliary proteins (the ciliome) in the pathogenesis of CHD. Previous studies have suggested an association between ciliary dysfunction and CHD with heterotaxy, a birth defect characterized by discordant cardiac, lung, and visceral organ situs resulting from aberrant embryonic left-right patterning. This study will use next-generation sequencing technologies to analyze whole exomes of CHD patients to identify disease-causing polymorphisms, specifically in the genes comprising the ciliome. These findings will likely shed light on the complex genetics of CHD and may lead to new diagnostic genetic tests that can help identify patients, specifically infants and children, at high risk for ciliary disorders.

Anticipated Duration of Project

1/1/2011 - 12/31/2014

Project Overview

Heterotaxy is a birth defect characterized by discordant cardiac, lung, and visceral organ situs resulting from aberrant embryonic left-right patterning. Patients with heterotaxy exhibit some of the most complex congenital heart diseases (CHD). Such patients usually require risky surgical repairs, with high rates of postsurgical morbidity, and often develop postsurgical respiratory complications, sometimes becoming ventilator dependent. As mucociliary clearance in the airway requires ciliary motion, respiratory complications and poor outcome could arise from undiagnosed ciliary dysfunction, such as that associated with primary ciliary dyskinesia (PCD). This possibility is particularly compelling for heterotaxy patients, as motile cilia are also required for the specification of laterality during embryonic development. To investigate the role of cilia defects in CHD, we recently initiated a clinical study that recruited patients with complex CHD associated with heterotaxy. From a cohort of 42 patients, 41 percent were observed to exhibit ciliary dysfunction. These results strongly suggest a role for the cilium in human CHD and heterotaxy. We hypothesize that sequence variants in genes required for cilia structure and function (i.e., genes of the ciliome) may play a central role in CHD pathogenesis. To test this hypothesis, we will use whole exome capture and massively parallel sequencing to scan for mutations in the coding exons of the human genome, with particular focus on the ciliome genes. The enriched genomic deoxyribonucleic acid (DNA) recovered from sequence capture will be amplified and sequenced with 100 X coverage using an AB SOLiD™ sequencing instrument. Of particular interest are nonsynonymous variants leading to missense or nonsense mutations and splicing defect variants or small insertions/deletions that may cause frameshifts. Mutations causing CHD are expected to be rare in the unaffected population, with allele frequencies < 1 percent. High priority single nucleotide polymorphisms (SNPs) identified from this analysis will be validated by direct sequencing of patient genomic DNA and further interrogated across datasets available in the public domain, such as from the 1,000 Human Genomes Project. The findings from this first patient cohort will be validated with similar analysis of a replication cohort of an additional 200 patients to be recruited from Children’s Hospital of Pittsburgh of UPMC. Overall, these studies may yield novel insights into the complex genetics of CHD.
Results from this study may suggest changes in the standard of care that may improve the long-term prognosis of heterotaxy patients with complex CHD.

Specific Aims:

Aim 1. We will identify possible disease-causing sequence variants using whole exome capture and next-generation sequencing in 50 patients with complex CHD and heterotaxy.

Aim 2. Sequence variants recovered in the ciliome and CHD genes will be interrogated using online repositories, such as dbSNP or HapMap, to identify those that are rare variants or mutations. These sequence variants will be validated by capillary sequencing of the patient DNA and further interrogated against sequencing data from the 1,000 Human Genome Project and other publically available human exome/whole genome datasets.

Aim 3. We will validate rare disease-causing sequence variants recovered in the CHD patients. Validation will occur by conducting exome capture and SOLiD sequencing of a replication cohort of 200 CHD patients to be recruited from Children’s Hospital of Pittsburgh of UPMC.

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Expected Research Outcomes and Benefits

Our study seeks to demonstrate a link between ciliary dysfunction, mutations involving genes essential for cilia structure and function, and CHD. These findings may suggest a broad role for the cilium in a wide spectrum of CHD previously thought to have no unifying etiology. This hypothesis, if validated, may suggest changes in the standard of care that could improve the postsurgical outcomes and long-term prognosis of patients with complex CHD. In addition, results from our DNA analyses may provide the basis for the development of diagnostic genetic tests that can help identify patients at high risk for PCD among patients with complex CHD. These data could also suggest changes in the standard of care to include presurgical screening for PCD and instituting appropriate preoperative and postoperative pulmonary therapies, which could improve the long-term prognosis of heterotaxy patients with complex CHD. Overall, these studies may yield novel insights into the complex genetics of CHD and provide strategies for improving the long-term outcomes for our most vulnerable patient population—infants and young children with complex CHD.
Summary of Research Completed

Progress – Aims 1 and 2:

To investigate the genetic etiology of congenital heart disease (CHD), we are undertaking large-scale next generation sequencing of deoxyribonucleic acid (DNA) from patients with CHD associated with heterotaxy (the randomization of left-right patterning of visceral organ asymmetry). Our studies have initially focused on the role of the cilia and the contribution of mutations in the 13 genes known to cause the cilia-related disorder known as primary ciliary dyskinesia (PCD). PCD patients can have heterotaxy and CHD; thus, we hypothesize that mutations in PCD-related genes may play a role in CHD associated with heterotaxy.

In our first cohort of CHD patients with heterotaxy, 42 percent of patients with CHD associated with heterotaxy had ciliary dysfunction (CD). Exome sequencing of 26 patients in this cohort showed 13 with CD and 13 without CD. We also sequenced 12 PCD patients as disease controls and 10 healthy individuals as healthy controls. The exome sequencing data obtained from these 48 individuals were analyzed using the bioinformatics pipeline we developed in the past year. This analysis includes filtering the exome data to remove common nondisease causing variants and filtering of variants in dbSNP131, 1000Genomes (http://www.1000genomes.org), and the National Heart, Lung, and Blood Institute (NHLBI) Grand Opportunity Exome Sequencing Project (ESP) exome databases. Unique variants identified after this bioinformatic analysis were validated with Sanger capillary sequencing.

From this analysis, we found that CHD patients with CD are highly enriched for mutations in the 13 genes known to cause PCD (see table below). The incidence of mutations was similar to that seen in patients with PCD. In contrast, CHD patients with CD or healthy controls had very low incidence of novel mutations in the 13 PCD genes (see table below). The results of this analysis have been published in Circulation, Nakhleh et al. (2012).

We completed exome sequencing on an additional 41 patients with CHD. Coding variants were identified using the same bioinformatics pipeline described above to filter out all common variants in the human genome. Rare coding variants found were further analyzed using Polyphen and SIFT programs to determine which of these variants are likely to be deleterious to protein function. We also checked these rare coding variants against the Human Genome Mutation Database (HGMD) to determine whether any are known disease-causing mutations. We successfully identified known disease-causing mutations in five patients. Among the remaining patients, many were found to have candidate disease-causing mutations. Of particular interest, one heterotaxy patient with CD (patient 9002) was found to have one of the most common pathogenic DNAI1 (IVS1+2_3insT ) mutations known to cause PCD. This mutation was found in heterozygosity; but, as both PCD and heterotaxy are recessive disorders, our observation would suggest a multigenic etiology for disease (i.e., the prediction is that disease may arise from the presence of a second disease-causing mutation in another cilia-related gene). Consistent with this hypothesis, this patient also was found to have a novel coding variant in DNAH6, a highly conserved motor dynein gene not previously known to cause human
disease. Interestingly, DNAH6 novel coding variants were found in two CD-heterotaxy patients and one PCD patient, but not in any heterotaxy patient without CD, nor in any of the healthy controls. Using short hairpin ribonucleic acid (shRNA) gene knockdown, we demonstrated that loss of DNAH6 can cause airway cilia dysmotility in human airway epithelial cells. In-situ hybridization analysis showed that DNAH6 is specifically expressed in the mouse E8.5 embryonic node, consistent with a role in left-right patterning. Using zebrafish embryos, we showed that Dnah6 morpholino knockdown can cause heterotaxy, as exemplified by randomized orientation of heart tube and gut looping. By immunostaining and videomicroscopy, we showed that Dnah6 knockdown disrupts ciliary motility and also fluid flow in Kuppfer’s vesicles, the equivalent of the embryonic node in the zebrafish embryo.

Together these data suggest that DNAH6 plays a critical role in both airway cilia motility and left-right patterning. In addition, we performed double knockdown of DNAH6 and DNAI1 and found that the double-knockdown embryos can have significantly higher incidence of heterotaxy at morpholino doses that otherwise do not cause heterotaxy. These findings suggest a multigenic etiology for CHD associated with heterotaxy, a new paradigm for the genetic etiology of CHD. This study is being prepared for publication.
Table 1. Novel Coding Variants (NCVs) in Heterotaxy and PCD Patients

<table>
<thead>
<tr>
<th>Patient Type</th>
<th>Total No. Subjects</th>
<th>No. with NCVs (%)</th>
<th>No. NCVs</th>
<th>NCVs Per Subject</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterotaxy with CD</td>
<td>13</td>
<td>7 (54%)</td>
<td>10</td>
<td>0.769</td>
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<tr>
<td></td>
<td></td>
<td>p=0.019</td>
<td></td>
<td>p=0.011</td>
</tr>
<tr>
<td>Heterotaxy without CD</td>
<td>12</td>
<td>3 (25%)</td>
<td>5</td>
<td>0.417</td>
</tr>
<tr>
<td>PCD</td>
<td>10</td>
<td>6 (60%)</td>
<td>10</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.03</td>
<td></td>
<td>p=0.006</td>
</tr>
<tr>
<td>Healthy Controls</td>
<td>13</td>
<td>1 (8%)</td>
<td>1</td>
<td>0.077</td>
</tr>
</tbody>
</table>

 p-value <0.025 was considered statistically significant for each comparison based on the Bonferroni correction. The Fisher’s exact test and the Kruskal Wallis test were used for comparisons of the presence and number of NCVs in the four groups, and both yielded significant results (P=0.021 and P=0.024, respectively).

Publications:


Research Project 3: Project Title and Purpose

Breaking Metabolic Symbiosis in Tumors: A New Cancer Treatment Paradigm - As cancer cells grow and evolve into tumors, they need specific nutrients and oxygen to fuel cellular metabolism, namely, through mitochondrial oxidative phosphorylation and glycolysis. This project will test the hypothesis that those cancer cells with the highest metabolic flexibility are most successful at developing tumors. In a tumor, the populations of cells farthest from an oxygen supply produce energy through glycolysis and create lactate, a waste product, which is used by the more oxygenated cell population to produce energy by oxidative phosphorylation. This project will examine how this metabolic symbiosis drives rapid tumor growth.
Understanding how this interdependent metabolic sharing of carbon sources occurs in a growing...
tumor and learning how to break the cycle will enable improved therapeutic approaches to cancer.

**Anticipated Duration of Project**

1/1/2011 - 12/31/2012

**Project Overview**

Cancer cells require nutrients to grow. Finding out what they ingest and how they grow remains one of the frontiers of cancer biology. Over 70 years ago, Otto Warburg found that tumor cells enjoyed consuming large amounts of glucose through glycolysis, even when supplied an abundance of oxygen. He and his team surmised that the mitochondria of tumor cells were broken and, therefore, could not perform oxidative phosphorylation (OXPHOS). This idea was later proven incorrect, as many tumor cells do show significant respiration and mitochondrial function is vital for their growth. However, one of the most important technologies for identifying metastatic tumors, using positron emission tomography (PET) imaging of F\(^{18}\)-labeled deoxyglucose, clearly indicates that metastatic tumor cells have an increased glucose uptake. Furthermore, the identification of mitochondrial deoxyribonucleic acid (mtDNA) mutation accumulation in a large number of different types of tumors suggests that mitochondrial dysfunction does occur in tumor cells. This project will test the hypothesis that tumor cells display remarkable metabolic flexibility and, depending on environmental cues, can evolve into one of two populations of tumor cells: those that are highly glycolytic at the hypoxic tumor core or those with robust mitochondrial oxidative phosphorylation, if well oxygenated through a vascular bed. Viewing tumor development and metastasis in this manner demands a two-pronged approach to kill both populations of cells by altering metabolism. This project will directly test this metabolic symbiotic model of tumor development through three interdependent aims. First, carbon-source preference and cellular bioenergetics will be examined in several panels of breast, lung, and melanoma tumor cell lines. Specific inhibitors of these processes will be used to interrupt cellular bioenergetics and to examine subsequent effects on cell growth. Second, expression of key genes and proteins controlling glycolysis and OXPHOS will be correlated to the metabolic potential of these cells. The third aim will translate these *in vitro* experiments to human cell xenograft models and primary tumor analyses for direct proof of two populations of metabolically distinct tumor cells. This final aim will also test the hypothesis that mitochondrial mutations arise in the hypoxic glycolytic portion of the tumor.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Cancer killed more than 500,000 people in the United States last year and is the cause of death of about one in four Americans. In addition, Pennsylvania lost almost 29,000 citizens to cancer in 2009. Cancer is characterized by uncontrolled cell growth and lack of cell death, and tumor growth is fueled by cellular bioenergetics. Attacking this source of energy alone or in combination with other anti-cancer strategies will help to slow the growth of tumors. This project will directly test the hypothesis that tumor development is mediated by the production of two interdependent cell populations that arise from a single cell type. These two cell populations, pro-glycolytic and pro-OXPHOS, use metabolic symbiosis to share carbon sources to fuel their growth. The hypoxic tumor cell population, which is deprived of oxygen, uses glucose through glycolysis to generate adenosine triphosphate (ATP). The waste product from this reaction, lactate, is then used to fuel the oxygenated cell population to drive OXPHOS. This project will: (1) characterize carbon source preferences for cellular growth using both bioenergetic pathways; (2) show how specific inhibitors of carbon source utilization, glycolysis, or OXPHOS can limit ATP production and subsequent cell growth; (3) characterize and validate gene and protein markers for glycolysis and OXPHOS; (4) provide direct evidence of two cellular populations within a human xenograft model and primary human tumors; and (5) demonstrate that mtDNA mutations arise in the glycolytic, oxygen-poor regions of the tumor. This project will benefit the cancer biology field by providing validated markers of tumor metabolism and logical targets for inhibition to slow tumor growth. The long-term benefit of this project will allow new chemotherapeutic approaches that specifically attack cellular bioenergetics to be added to the arsenal of anti-tumor therapies in the treatment of human cancer.

Summary of Research Completed

1. Analysis of carbon source preference in head and neck cancer and cervical cancer cell lines using the Biolog assay (Aim 1.1). We have completed an initial analysis of carbon source utilization by several head and neck tumor cell lines, which indicate that these cells have a high propensity to metabolize glucose and various sugars, but a low capacity to metabolize lactate (Figure 1). Cal33 cells demonstrated the greatest rates of metabolism for maltriose, D-maltose, alpha-D-glucose, fructose, uridine, and lactic acid, and metabolized lactic acid at 37 percent of the glucose metabolism rate. MDA-686LN cells were able to metabolize a greater variety of substrates, but the metabolites that were consumed at the fastest rates were similar to those metabolized by Cal33 cells. MDA-686LN cell metabolism of lactic acid occurred at 11 percent of the rate of glucose metabolism (data not shown). In addition, SiHa cervical cancer cell metabolism was assessed, as SiHa cells have been reported to consume lactate. SiHa cells were found to metabolize lactate at 31 percent of the rate of glucose metabolism (Figure 1). These data give some support for the metabolic symbiosis hypothesis in which tumor cells consume lactate to grow. This concept was reviewed in detail by Erica Nakajima (see publication No. 3 below).
2. Western analysis of several metabolic enzymes in head and neck and cervical tumor cell lines (Aim 2.1) (Figure 2). Lactate dehydrogenase-alpha (LDH-A) expression in SiHa cells cultured in lactate was significantly decreased in comparison to cells cultured in glucose. This result is in agreement with our predictions, as the LDH-A subunit promotes the conversion of pyruvate to lactate. The lack of a similar response in the head and neck cancer cell lines reflects their reduced ability to proliferate in lactate as compared to SiHa cells. Unexpectedly, pyruvate dehydrogenase kinase 1 (PDHK1) expression was increased in Cal33 and MDA-686LN cells cultured in lactate. PDHK1 inhibits the conversion of pyruvate to acetyl coenzyme A (CoA) by phosphorylating pyruvate dehydrogenase (PDH), thereby creating a build-up of pyruvate that is then converted to lactate. PDH expression was increased in Cal33 cells cultured in lactate, which is in agreement with our expectations. It is possible that elevated expression of PDH is triggering expression of PDHK1 (Figure 2).

3. Analysis of mitochondrial DNA (mtDNA) damage and OXPHOS changes after oxidative stress and alkylating agents (Aim 3.1.1). We addressed the question of whether persistent mtDNA damage causes a loss of oxidative phosphorylation by treating mouse embryonic fibroblasts with hydrogen peroxide (H$_2$O$_2$) or the alkylating agent methyl methanesulfonate (MMS) and measuring several endpoints, including mtDNA damage and repair rates using quantitative polymerase chain reaction (QPCR), levels of mitochondrial- and nuclear-encoded proteins using western analysis, and a pharmacologic profile of mitochondria using the Seahorse Extracellular Flux Analyzer. We found that a 60-minute treatment with H$_2$O$_2$ causes persistent mtDNA lesions, mtDNA loss, decreased levels of a nuclear-encoded mitochondrial subunit, loss of ATP-linked oxidative phosphorylation, and loss of total reserve capacity. Conversely, a 60-minute treatment with 2 mM MMS causes persistent mtDNA lesions but no mtDNA loss, no decrease in levels of a nuclear-encoded mitochondrial subunit, and no mitochondrial dysfunction. These results suggest that persistent mtDNA damage is not sufficient to cause mitochondrial dysfunction. These results were recently published (see publication No. 1 below).

4. Western analysis on primary tumor tissue to demonstrate metabolic symbiosis (Aim 3.1.2). To investigate whether melanoma tumors exhibit metabolic symbiosis, we looked at several protein markers of glycolysis (LDH-A, HIF-1a), OXPHOS (ATP5A1, LDH-B), and lactate exchange (MCT1, MCT2). We performed immunohistochemistry studies of a nevus\textgreater{}melanoma progression tumor microarray (TMA). LDH-A is expressed in nevic melanocytes and in primary and metastatic melanomas, but its levels are higher in primary thick melanomas. Data indicate increased expression of LDH-A with progression from nevi to advanced melanoma (Poisson model for trend p<0.001). Since it has been reported that hypoxia-inducible factor (HIF)-1a increases LDH-A expression, we also determined HIF-1a expression in the nevus\textgreater{}melanoma TMA. In agreement with the data of a previous report, HIF-1a was expressed in primary melanoma and increased with progression from nevi to advanced melanoma, especially lymph node metastases, although overall expression was low (H-score <100; Poisson model for trend p<0.001). To obtain experimental evidence that OXPHOS is elevated in melanoma tumors, we used an antibody to ATP5A1, a mitochondria-encoded subunit of ATP synthase, as a “marker” for OXPHOS to probe the nevus\textgreater{}melanoma progression TMA. In addition, we used an antibody to LDH-B, which encodes all four subunits of LDH1 that converts lactate to pyruvate. ATP5A1 expression was detected prevalently in the cytoplasm of melanoma cells. LDH-B, but not ATP5A1, expression was significantly increased in advanced melanomas compared with nevi.
(Poisson model for trend p<0.001 and p=0.098, respectively). However, ATP5A1 expression was significantly increased in both primary and metastatic melanomas compared with nevi (Figure 3). We have shown that OXPHOS is important for melanoma in addition to glycolysis. Mouse models have shown that cancer cells that are located in different areas of the tumor preferentially use one metabolic source over the other, depending on environmental cues. In fact, lactate produced by glycolytic cells is extruded from these cells and is taken up by OXPHOS-dependent cancer cells via membrane protein monocarboxylate transporters (MCTs), which transport lactate, pyruvate, and ketone bodies via an ATP-independent passive diffusion that follows substrate gradients and H+ transport. Given the lack of information regarding expression of MCT1 and MCT4 in melanoma tumor tissues, and the availability of highly specific small molecule MCT inhibitors (several of which are in early clinical development), we became interested in studying the expression of MCT1 and MCT4 in the nevus>melanoma TMA. Our TMA analysis revealed that MCT1 expression was primarily membranous, while MCT4 expression was mostly cytoplasmic. Expression of both MCT1 and MCT4 demonstrated an increase with progression from nevi to advanced melanoma (Poisson model for trend p<0.001 for MCT1 and MCT4). These results are being written up for publication.

5. Initial imaging experiments with the IR-2-DG on cells grown in culture and in a xenograft model (Aim 3.2). We are using three fluorogenic reagents to visualize tumor growth and metabolic symbiosis in vivo in human tumor mouse xenografts (Figure 4). To track tumor growth in real time, we have examined FP635-expressing UMSC221 head and neck cancer cells. To track glucose utilization, we are using IR800-2-deoxyglucose (IR-2-DG) in Lu1205 melanoma cells; to detect hypoxic cells, we are using hypoxisense at 685 nm (not shown). Fluorescence imaging is being done on live mice using an FMT 2500 Quantitative Tomography In Vivo Imaging System (PerkinElmer Inc., Boston, MA). Fluorescence molecular tomography (FMT) imaging allows visualization of fluorescent signal in 3D at a depth of up to 7-14 cm, and a resolution of 0.3 mm. Four lasers in the FMT induce excitation with wavelengths of 635, 680, 740, and 790 nm. The FMT is being used to generate a 3D image of the tumor via the detection of FP635 (Figure 4B). We can also visualize vertical and horizontal slices of the tumor to identify areas of the most severe hypoxia and greatest glucose uptake. Differences in the intensity of IR-2-DG signal will be analyzed using a signed rank test. Mice are imaged after IR-2-DG administration (Figure 4A).

6. The effects of inhibitors on glycolysis and OXPHOS as analyzed by cell growth, Seahorse XF24 Flux analyzer, and ATP (Aim 1.2). We have been studying the effect of various metabolic inhibitors on rates of OXPHOS and glycolysis and have made the surprising finding that 80 percent to 100 percent of OXPHOS can be blocked with the carnitine palmitoyltransferase 1 (CPT1) inhibitor, etomoxir (Figure 5). CPT1 is essential for the uptake of fatty acids into the mitochondria for beta-oxidation. We are testing the hypothesis that cells turn glucose into citrate in the mitochondria, which is shuttled out of the mitochondria to be used to make fatty acids and lipids. These lipids represent energy stores that can then be burned later in the mitochondria.

Relevant publications supported by PA Department of Health (7/1/11-6/30/12):
5. Green DR and Van Houten B. SnapShot: Mitochondrial quality control. Cell. 2011 Nov 11;147(4):950, 950.e1. PubMed PMID: 22078889. Note: These types of articles do not allow acknowledgements, but PA CURE support was used for the development of this paper.

![Graph](https://example.com/graph.png)

Figure 1. Biolog assay. This assay measures relative growth in the indicated carbon sources.
Figure 2. Western blots of key metabolic enzymes. Cells were grown in media containing glutamine pyruvate (DMEM) or media with 25 mM glucose (Glu) or 10 mM lactate (Lac). Red squares indicate significant changes.

Figure 3. Immunohistochemistry of a protein marker of OXPHOS in melanoma tumors. Human melanomas were compared to nevi for their expression of a protein subunit of complex V, which is involved in making ATP in the mitochondria by oxidative phosphorylation.
Figure 4. Preliminary in vivo imaging studies. (A) Subcutaneous imaging of Lu1205 melanoma xenograft uptake of IR-2-DG using the IVIS Imaging System. (B) UMS221 head and neck cancer cells expressing FP635 were injected peritoneally into an athymic nu-/nu-mouse and imaged via FMT after 5 days.

Figure 5. Decrease in OXPHOS by inhibition of fatty acid oxidation. Etomoxir, ETO (100 μM) was injected into the Seahorse bioanalyzer, and the amount of oxygen consumed by two different melanoma cell lines was measured.
Research Project 4: Project Title and Purpose

Prostate Cancer Vaccine Clinical Trials - This project encompasses investigator-initiated clinical trials that explore the safety and efficacy of novel therapeutic cancer vaccines, with the overarching goal of improving outcomes of patients with cancer. Aim 1 includes therapeutic trials of novel cancer vaccines based on polarized alpha dendritic cells (αDC1s) for the treatment of prostate cancer, and Aim 2 examines the efficacy of novel tumor peptide-based cancer vaccines for the treatment of prostate cancer.

Anticipated Duration of Project

1/1/2011 - 12/31/2012

Project Overview

The overall goal of this project is to examine the safety and efficacy of novel therapeutic cancer vaccines designed to improve patient outcomes. Aim 1 will examine αDC1-based vaccines loaded with an allogeneic prostate cell line (LNCap) in combination with androgen ablation (AA) in patients with recurrent prostate cancer who have failed local therapy and have no measurable metastasis but have a rising prostate specific antigen (PSA), with a doubling time of less than 12 months (UPCI 06-070). The primary efficacy objective is to evaluate the effect of the αDC1 vaccine on time to PSA progression compared to AA alone.

Aim 2 will examine trials of novel tumor peptide-based vaccines, including safety and efficacy studies of: (1) a vaccine targeted against mucin-1 (MUC-1), given in conjunction with poly-ICLC (polynosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose) in patients with recurrent and/or advanced prostate cancer (UPCI 05-086). The vaccine approaches described in this project offer immunotherapeutic potential to reduce the risk of tumor recurrence, which may translate into improved patient survival.

Principal Investigator

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Other Participating Researchers

None
Expected Research Outcomes and Benefits

Aim 1:
**UPCI 06-070:** This study aims to develop a novel vaccine to treat men with recurrent prostate cancer who have relapsed following surgery and/or radiation and now have a rising PSA, with a PSA doubling time of less than 12 months. This target patient population is ideal for an immunologic intervention, given that: (1) this is the largest sub-population of men with prostate cancer in the United States, (2) there is no standard treatment for this sub-group, and (3) this sub-group has minimal systemic disease burden. Therefore, there is great potential to improve patient outcomes in this group through the development of novel therapies.

Aim 2:
**UPCI 05-086:** The goal of this study is to develop a safe and effective peptide-based vaccine for men with advanced prostate cancer, which could potentially prolong survival with minimal side effects.

Summary of Research Completed

As reported previously, the phase I portion of the UPCI 05-086 trial has been completed, and results were presented at the 2011 American Society of Clinical Oncology (ASCO) meeting (and outlined in last year’s progress report). Since then, we have received approval from the Pennsylvania Department of Health to focus solely on the 06-070 trial for the remainder of the project funding period. In addition, we received approval to transfer leadership of this project to Leonard Appleman, MD, as Gurkamal Chatta, MD, has left the University of Pittsburgh Cancer Institute (UPCI). Dr. Appleman is a medical oncologist and expert in the field of genitourinary malignancies and has taken over the role of principal investigator of the 06-070 trial at UPCI. While this transition of leadership has inevitably caused delays in the progress of the 06-070 trial, the accrual process is actively underway and is expected to be completed by the end of the year. Once safety and efficacy analyses of this novel therapeutic regimen are complete, results will be submitted to a peer-reviewed journal for publication.

Research Project 5: Project Title and Purpose

**Melanoma Vaccine Clinical Trial** - This project is an investigator-initiated clinical trial that explores the safety and efficacy of novel therapeutic cancer vaccines based on advances made at the University of Pittsburgh in the generation of durably polarized alpha dendritic cells (αDC1s) for the treatment of melanoma.

Anticipated Duration of Project

1/1/2011 – 12/31/2012
Project Overview

The overall goal of this project is to examine the safety and efficacy of novel therapeutic cancer vaccines designed to improve patient outcomes. We will examine multi-epitope anti-melanoma vaccines based on polarized alpha dendritic cells (αDC1s), comparing αDC1s to standard DCs in patients with advanced (stage III/IV) melanoma (UPCI 03-118). This study was designed to evaluate the ability of both types of DCs to induce different sets of chemokine receptors on tumor-specific CD8+ T cells.

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Expected Research Outcomes and Benefits

The planned studies represent the first human application of prolonged infusion of antigen-loaded DCs. The success of these studies will allow the reduction of the DC dose used in similar applications in patients with melanoma and other types of cancer, thereby reducing treatment costs and increasing its effectiveness.

Summary of Research Completed

A total of 22 patients have been treated on this trial, which was closed to accrual in January 2012. Evaluation of immunologic endpoints is ongoing. Several patients among the initial cohort have exhibited antitumor effects and autoimmune anti-pigmentary immune responses that are
interpreted as early proof of concept, but the overall survival and time-to-progression (TTP) analyses did not reveal significant differences between the study groups (data not shown).

Interpretation of trial results has been made difficult by an observation of systematically lower than anticipated levels of interleukin (IL)-12 produced by vaccines (average is up to 10 times lower than levels seen in our preclinical studies and with a concurrent melanoma trial recently completed by our collaborators in Heidelberg and Essen, Germany). Our partially completed analysis suggests that a cause for this discrepancy might have been a suboptimal combination of dendritic cell (DC) culture period duration with the concentration of maturation-inducing factors and the type of culture medium used (the original medium used to develop the vaccine was discontinued). Our prospective work will involve modification of DC culture conditions in the currently available medium. Demonstration of the feasibility of prolonged cannulation of lymphatic vessels constitutes the major novelty of the study.

Research Project 6: Project Title and Purpose

_Glioma Vaccine Clinical Trials_ - The University of Pittsburgh Cancer Institute’s Clinical Research Service (CRS) provides valuable resources for researchers seeking to improve patient care standards and treatment efficacy. This project encompasses investigator-initiated clinical trials that explore the safety and efficacy of novel tumor peptide-based cancer vaccines for the treatment of low-grade glioma (LGG).

Anticipated Duration of Project

1/1/2011 – 12/31/2012

Project Overview

We will examine trials of novel tumor peptide-based vaccines, including safety and efficacy studies of: vaccinations with glioma-associated antigen (GAA) peptides emulsified in vaccine adjuvant Montanide ISA-51 and in combination with polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (poly-ICLC) in patients with either newly diagnosed low-grade glioma (LGG) with high risk factors for recurrence (UPCI 07-057) or in patients with recurrent LGG (UPCI 08-135). The vaccine approaches described in this project offer immunotherapeutic potential to reduce the risk of tumor recurrence, which may translate into improved patient survival.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Adults with supratentorial LGG have a significant risk of tumor progression following treatment with surgery or surgery followed by radiation therapy (RT), and most LGG patients eventually die of the disease. The vaccine approach described in these trials offers both immunotherapeutic and immunoprophylactic potential to reduce the risk of tumor recurrence, which could translate into improved survival. The slower growth rate of LGG (in contrast to malignant gliomas) should allow sufficient time to administer multiple immunizations, potentially leading to the induction of high levels of glioma-associated antigen-specific immunity.

Summary of Research Completed

UPCI 07-057:
Objectives: Primary objectives are to determine immunological activity and safety of the regimen. Such data will serve as bases to decide whether a larger follow-up study is warranted. To assess induction of GAA-specific T-cell response, we will determine the response rate and magnitude of immune response in post-vaccine peripheral blood mononuclear cells (PBMC) against the GAA peptides in response to this form of vaccine using interferon (IFN)-γ-enzyme-linked immuno-spot (ELISPOT) and tetramer assays. For safety, the incidence and severity of adverse events (AE) associated with the vaccine regime will be assessed, with an early stopping rule based on the frequency of regimen limiting toxicity (RLT). Other exploratory objectives include: (1) clinical response, (2) radiological response, (3) two-year progression-free survival (PFS), and (4) evaluation of tumor tissues for biological correlates.

Methods: Eligible patients are human leukocyte antigen (HLA)-A2+ patients (age ≥18 years) with histologically diagnosed supratentorial World Health Organization (WHO) grade II astrocytoma or oligoastrocytoma with “high-risk” factors, defined as: (1) age ≥ 40 with any extent of resection, (2) age 18-39 with incomplete resection (post-op magnetic resonance imaging [MRI] showing >1 cm residual disease, based on the maximum dimension of residual T2 or fluid-attenuated inversion-recovery [FLAIR] abnormality from the edge of the surgical cavity either laterally, antero-posteriorly, or supero-inferiorly], or (3) tumor size ≥ 4 cm (any age).

Eligible patients are stratified based on whether they have undergone prior radiation therapy. Cohort 1 includes patients who have undergone surgery or biopsy alone (no postoperative radiation or chemotherapy) and have a baseline MRI scan (within four weeks of the first vaccine) that shows stable disease or regression (no progression from the initial surgery/biopsy). Cohort 2 includes patients who received surgery or biopsy and radiation therapy (RT), which was completed ≥ six months prior to enrollment, and have a stable MRI scan (no progression after RT). Prior chemotherapy excludes patients from both cohorts. The sample size was originally
nine patients per cohort; however, the sample size for Cohort 1 has been expanded to a maximum of 18 because of a high accrual rate in this cohort.

All participants must have discontinued dexamethasone or similar corticosteroid medications at least four weeks before administration of the first vaccine. Participants are treated with subcutaneous (s.c.) injections of GAA/Tt-peptide vaccines on an outpatient basis on weeks 0, 3, 6, 9, 12, 15, 18, and 21; poly-ICLC is administered (20 mg/kg intramuscularly [i.m.]) on the day of and again four days after vaccination. See Table 1 and Schema 1 below for a treatment schedule and list of antigens. Participants are evaluated for any possible AE, RLT, and clinical response by clinic visits and MRI. PBMC samples are drawn at pre-vaccine and post-vaccine time points to evaluate immune responses.

**Results:** We have enrolled two new patients in Cohort 1, and we have continued treating the other patients who were enrolled previously.

As of June 11, 2012 twelve patients have received at least one vaccine in Cohort 1. Nine of them have completed the initial course of eight vaccines. Of the three patients who have not completed the eight-vaccine course to date, one was taken off due to rapid tumor progression; another has safely received two vaccines and is still on the study. The third was taken off due to the occurrence of Common Terminology Criteria for Adverse Events (CTCAE) Grade 3 fever following the seventh vaccine, which is a dose-limiting toxicity (DLT). This is the only patient who demonstrated DLT; the symptom had subsided by the next day, after administration of an over-the-counter non-steroidal anti-inflammatory drug. Among the nine patients who have completed the initial eight vaccines, five have been taken off due to radiologic or symptomatic progression.

Most patients experienced mild and transient CTCAE Grade 1 or 2 flu-like symptoms, including low-grade fever, chills, myalgia, headache, and fatigue. One patient is currently enrolled in Cohort 2 (patients with prior RT). This patient completed the initial eight vaccines with stable disease and has received five booster vaccines with a stable disease condition.

Immune response analyses are performed after each patient completes the treatment regimen. ELISPOT assays have been completed in seven Cohort 1 patients and in the single patient in Cohort 2. These demonstrated robust and sustained interferon (IFN)-γ (type-1) responses against at least three of the GAA epitopes in all cases, while interleukin (IL)-5 (type-2) responses were absent or transient in all cases. The magnitude of the IFN-γ ELISPOT responses in this study is significantly higher than that observed in our previous phase I/II study in patients with recurrent high-grade glioma (Figure 1). Although evaluation of progression-free survival would require a longer observation period, our preliminary results demonstrate the regimen in these patients is well tolerated and induces a robust type-1 anti-GAA T-cell response. We have closed enrollment in this study; a new study, UPCI 11-136, which also targets the same patient populations but does not include the eligibility restriction for HLA-A2+ patients, has received IRB approval.

**UPCI 08-135:**
**Objectives:** This is a pilot vaccine study in adults with recurrent WHO grade II glioma. The overall objective is to collect immunological and safety data that will be used to decide whether a
larger study of clinical efficacy is warranted in these patients. All patients on the study will be followed for a minimum of two years to determine actual two-year overall survival (OS), as well as six-month and two-year progression-free survival (PFS) rates in an exploratory manner. Methods for evaluation of the primary and exploratory endpoints are the same as those delineated in UPCI 07-057.

**Methods:** Eligible patients are HLA-A2+ patients (age ≥18 years) with histologically diagnosed supratentorial WHO grade II glioma with recurrence. Patients have to be off steroids for four weeks before initiation of vaccines and must have a lymphocyte count of 400 microliters (µL) or more. The sample size for this study is nine patients. Participants are treated with s.c. injections of GAA/TT-vaccines on an outpatient basis on weeks 0, 3, 6, 9, 12, 15, 18, and 21; poly-ICLC is administered (20 mg/kg i.m.) on the day of and again four days after each vaccine. See Table 1 and Schema 2 below for a treatment schedule and list of antigens. Participants are evaluated for any possible AE, RLT, or clinical response by clinic visits and MRI. PBMC samples are drawn at pre-vaccine and post-vaccine time points to evaluate immune responses.

**Results:** We enrolled two new patients and continued treating patients who were enrolled previously. As of June 11, 2012, 10 patients have received at least one vaccine. Of those, nine have completed the initial eight vaccines, despite the fact that all patients had recurrent LGG when they entered the study. Six of those nine patients have recurred to date.

One patient had to be taken off the study after four vaccines due to rapid tumor progression. The toxicity profile has been very similar to that of UPCI 07-057. There were no RLTs. To date, comprehensive immune response assessments have been completed in one patient (the one who completed the initial eight vaccines but recurred after two booster vaccines). This patient demonstrated positive IFN-γ responses in ELISPOT assays (data not shown). We have closed enrollment in this study; a new study, UPCI 11-136, which also targets the same patient populations but does not include the eligibility restriction for HLA-A2+ patients, has received IRB approval.

In summary, accrual for these trials has been going very well. The regimen has been tolerated very well in both studies, except for a single case in UPCI 07-057, who presented with Grade 3 fever.

Although further analyses of immune responses await treatment completion, our preliminary results demonstrate that the regimens in both studies are well tolerated and induce robust type-1 anti-GAA T-cell responses.

Study UPCI 11-136 will serve as a continuation of these studies that will further indicate the immunological activity and safety of these treatment regimens.
Table 1. List of antigen peptides used in UPCI 07-057 and UPCI 08-135 studies

<table>
<thead>
<tr>
<th>Antigen Peptide</th>
<th>Presented By:</th>
<th>Prevalence in HG / GIIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-13Rα2 345-353:1A9V</td>
<td>HLA-A2</td>
<td>&gt;80% / low</td>
</tr>
<tr>
<td>EphA2 883-891</td>
<td>HLA-A2</td>
<td>75-80% / 50%</td>
</tr>
<tr>
<td>Survivin 96-104:M2</td>
<td>HLA-A2</td>
<td>All astrocytoma (GII-IV)</td>
</tr>
<tr>
<td>WT1 126-134:Y1</td>
<td>HLA-A2</td>
<td>All astrocytoma (GII-IV)</td>
</tr>
<tr>
<td>Tetanus Toxoid (TetA830)</td>
<td>Pan-DR</td>
<td>(heterologous antigen)</td>
</tr>
</tbody>
</table>

HG; high grade (grade III-IV) glioma, GIIA; grade II astrocytoma

Schema 1. Treatment course for the UPCI 07-057 study

- No corticosteroid will be allowed within 4 weeks prior to the first vaccine. Baseline MRI and other screening procedures will be done within 4 weeks prior to the 1st vaccination.
- Vaccines: Peptide-vaccines Q3W (Wk 0-21) and i.m. poly-ICLC (on days 0 and 4 following each vaccination).
- PBMC for immune studies (Q3W: Wk 12-24); MRI (Wks 12 & 24).
- Additional vaccines and poly-ICLC (Q12W) if applicable.
Figure 1. Patients in UPCI 07-057 study demonstrate higher levels of EphA2-specific IFN-γ responses than patients with high-grade glioma in the UPCI 05-115 study. IFN-γ ELISPOT data for two of the antigen epitopes (i.e., IL-13Rα [345-3531A9V] and EphA2 [883-891]) are compared in the two studies, UPCI 07-057 for patients with low-grade glioma (LGG) and UPCI 05-115 for recurrent high grade glioma (HGG). Although the therapeutic regimens in these two studies are not the same, both studies employed two common GAA epitopes (i.e., IL-13Rα [345-3531A9V] and EphA2 [883-891]) and the comparison was made based on the IFN-γ ELISPOT data at Week 12 (following the initial 4 vaccinations) in both studies. P-values between the two groups for IL-13Rα [345-3531A9V] and EphA2 [883-891] are 0.111 and <0.001, respectively.

Schema 2 – Treatment course for the UPCI 08-135 study

No corticosteroid will be allowed within 4 wks prior to the first vaccine. Baseline MRI and other screening procedures will be done within 4 wks prior to the 1st vaccination

Vaccines: Peptide-vaccines Q3W (Wk 0-21) and i.m. poly-ICLC (on days 0 and 4 following each vaccination)

PBMC for immune studies (Q3W: Wk 12-24); MRI (Wks 12 & 24)

Additional vaccines and poly-ICLC (Q12W) if applicable