The Institute for Cancer Research

Annual Progress Report: 2012 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

The Institute for Cancer Research (formerly Fox Chase Cancer Center) received $2,176,686 in formula funds for the grant award period January 1, 2013 through December 31, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

*ABCC10 Mediated Drug Resistance in Breast Cancer* – A structurally distinct group of ATP Binding Cassette (ABC) drug efflux pumps, known as the Multidrug Resistance Proteins (MRPs), have been gaining increasing attention as sources of resistance. Our *in vitro* studies show that MRP7 (ABCC10) overexpression confers resistance to taxanes and vinca alkaloids. Excitingly, our recently developed *Abcc10*\(/^{−}\)/ knockout mice are highly sensitized to paclitaxel treatment, supporting the idea that changes in *Abcc10* expression are sufficient to influence drug resistance. The purpose of this research is to evaluate the role of ABCC10 as a determinant of the efficacy of clinically relevant breast cancer therapies.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

In 2009, over 200,000 women were diagnosed with breast cancer, with 40,000 women expected to die, making breast cancer the second most lethal cancer for women. Much of the mortality is associated with resistance to therapeutic agents. The taxanes docetaxel and paclitaxel have been mainstays of cancer treatment regimes, and resistance to chemotherapy with these agents has long been a major impediment to cancer treatment. A group of ATP Binding Cassette (ABC) drug efflux pumps, known as the Multidrug Resistance Proteins (MRPs), has been identified as a source of resistance. Our published *in vitro* studies show that MRP7 (ABCC10) overexpression confers resistance to taxanes. Recently, we showed that the *Abcc10*\(/^{−}\)/ knockout mice we developed are highly sensitized to paclitaxel treatment, supporting the hypothesis that changes in *Abcc10* expression are enough to influence drug resistance. ABCC10 is the only transporter for which this has been demonstrated in connection with taxane treatment. Despite sensitization to high levels of taxanes, no sensitization is observed at low doses. Therefore, therapeutic efficacy might be achievable with ABCC10 inhibition, and ABCC10 may be a good candidate for modulation in cancer treatment regimens. However, little is known regarding ABCC10
expression or if ABCC10 modulation can increase tumor sensitization despite its clear role in conferring drug resistance.

Our *central hypothesis* is that inhibition of ABCC10, will enhance breast cancer treatment. We propose the three following *specific aims*:

Aim 1. Define ABCC10 expression pattern in breast cancer.
Aim 2. Determine if Abcc10 inhibition alters drug resistance phenotypes in ErbB2 (Her2+) breast cancer.
Aim 3. Evaluate the role of ABCC10 as a determinant of the efficacy of clinically relevant combination therapies.

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**Expected Research Outcomes and Benefits**

There are several benefits and outcomes of the research. This research will give us an early indication of whether ABCC10 is a resistance factor for taxanes and if this protein can be modulated in breast cancer to sensitizes tumors *in vivo*. This research will also establish the expression levels of ABCC10 as expressed in various molecular subtypes of breast cancer to assess ABCC10 relevance to human cancer. Our goal is to extend the findings from this study to support subsequent clinical investigations. Perhaps, then treatment regimens can be modified in the future; for example tumors that express higher levels of ABCC10 might respond better to anthracycline or radiation therapy than taxanes. In short, ABCC10 may serve as a predictor of taxane response and therapies may be tailored to achieve maximal effectiveness through careful patient selection and potentially combinatorial therapies including tyrosine kinase inhibitors that specifically inhibit ErbB family members.

**Summary of Research Completed**

*Aim 2.1 Determine if Abcc10 inhibition alters drug resistance phenotypes in Her2+ (ErbB2) breast cancer.*

Quantitative Real-time PCR. We hypothesized that Abcc10 loss would result in the upregulation of other transporters therefore we compared the transporter levels in the MMTV-ErbB2:Abcc10−/−
and MMTV-ErbB2; Abcc10+/− lines. Total ribonucleic acid (RNA) was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s suggestions. RNA was reverse-transcribed using the moloney murine leukemia virus (M-MLV) reverse transcriptase and a mixture of anchored oligo-dT primers and random decamers. Aliquots of complementary deoxyribonucleic acid (cDNA) were used for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Cycling conditions were 95°C, 15 min followed by 40 cycles (95°C, 15 sec; 60°C, 60 sec). To compare transporter expression levels, all mean quantity data were normalized to an independent gene Ppib (cyclophilin B). We analyzed the gene expression levels of Abcc1, Abcb1b, Abcc4, Abcc5 and Abcg2 in 3 Abccc10−/− and 4 wild-type lines. We found that Abcc10 loss promotes reduced gene expression of Abcb1B, Abcc4 and Abcc5.

Mammary tumor cell lines originating from MMTV-ErbB2; Abcc10 null cells are sensitized to cytotoxic ABCC10 substrates.

We hypothesized since Abcc10 is a taxane transporter that its absence should provide increased sensitization to microtubule directed agents. We performed an analysis of phalloidin, 4,6-diamidino-2-phenylindole (DAPI), by fluorescence microscopy after treatment with various concentrations of a microtubule-stabilizing agent. Incubation with taxanes affected the morphology of Abcc10 null cells more dramatically than Abcc10 wild-type cells. We observed significant tubulin stabilization at 10 nM docetaxel in MMTV-ErbB2;Abcc10−/− cells, while MMTV-ErbB2;Abcc10+/− cells required a higher concentration (25 nM) for a similar effect. We performed a similar assessment of the impact of epothilone B, and found that 3 nM epothilone disrupted the microtubules of the MMTV-ErbB2;Abcc10−/− cells, whereas 10 nM epothilone B was required before the microtubules were visibly disrupted in the wild-type lines.

Aim 2.2 Does Abcc10 status affect taxane and epothilone B sensitivity in mammary orthotopic tumor assays?

We have completed treatment of MMTV-ErbB2;Abcc10+/− (n=2 lines) and MMTV-ErbB2; Abcc10+/− (n=2 lines) with docetaxel and compared to vehicle treated only. We observed that Abcc10 loss promotes increased docetaxel response, such that growth of tumors is delayed in the Abcc10−/− tumors compared to the wild-type tumors. Loss of Abcc10 also promoted survival. We also began to look at the impact of Abcc10 status on epothilone B. These studies are ongoing. Unfortunately, the initial mice we tested could not tolerate epothilone B, as a result we are now lowering the dose and frequency of administration.

Aim 3. Evaluate the role of ABCC10 as a determinant of the efficacy of clinically relevant combination therapies.

We have completed the vehicle only Cremophor/EtOH arm for the paclitaxel study, with 9 MMTV-Neu;Abcc10−/− and 7 MMTV-Neu;Abcc10+/− being currently analyzed for response to vehicle. We have injected 15 MMTV-Neu;Abcc10−/− mice and 9 wild-type mice for the paclitaxel only study. We are currently analyzing the data for these studies. Early data suggests that Abcc10 loss might sensitize tumors to paclitaxel, but not to the levels that Abcc10 loss sensitizes tumors to docetaxel.
Figure 1. Mammary tumor cell lines originating from MMTV-Neu;ABCC10⁻/⁻ accumulate more paclitaxel compared to MMTV-Neu;ABCC10⁺/⁺ cells. Accumulation of 0.1 μM [³H]-paclitaxel in Abcc10⁺/⁺ cells (WT, ▲) compared to the mammary tumor cell lines derived from Abcc10 disrupted mice (KO, ∆) was observed. Error bars denote mean ± SEM. Statistical significance was determined by two-sided Student’s t test.
Research Project 2: Project Title and Purpose

Quaternary Structure Dynamics in Protein Function, Disease, and Therapy – Protein structure dynamics are essential for protein function. Factors that modulate protein structure dynamics can contribute to disease pathology and therapeutic mechanisms. Our laboratory is focused on the role of quaternary structure dynamics in the control of protein function, the etiology of disease, and the discovery of small molecule therapeutics. This project uses disease-associated human proteins to correlate quaternary structure dynamics with protein function.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

Our recent research has revealed that at least one, and perhaps many, proteins participate in a dynamic quaternary structure equilibrium wherein alternate conformations of a monomer favor assembly to structurally and functionally distinct multimers. The position of this quaternary

Figure 2. Therapeutic effects of docetaxel on orthotopic mammary tumor model. Allograft model of SCID mice injected with Abcc10<sup>C</sup> (A, B) or wild-type (C, D) lines treated with docetaxel after tumor development (Δ, vehicle treated, ▲ docetaxel treated). Red lines (untreated) or blue lines (untreated) indicate progression of individual mice.
structure equilibrium provides a mechanism for physiologically relevant allosteric regulation of protein function, which we have called the morpheein model for allostery. Our initial work on this model focused on the protein porphobilinogen synthase (PBGS) whose dysfunction is related to the inborn error of metabolism (IEM) ALAD porphyria. The position of the PBGS equilibrium is shifted towards a low activity multimer for disease-associated variants, providing a structural basis for an IEM and an expanded view of conformational diseases. Most significantly, the position of the equilibrium can be modulated by small molecules for therapeutic advantage and/or to understand undesirable side-effects. This project expands our research to address the applicability of these concepts more broadly to protein function, to IEMs, and to cancer, with the overall goal of generating data that will lead to extramural support for new therapeutic approaches. AIM 1 expands our work with PBGS to the identification of novel octamer-stabilizing compounds that could function as therapeutics for the IEM ALAD porphyria. AIM 2 addresses the relationship between the morpheein model of allostery and the IEM phenylketonuria (PKU), which is most often caused by dysfunction of phenylalanine hydroxylase (PAH). We will address whether allosteric regulation of PAH must be dissociative. We will develop a high throughput screen to evaluate compound libraries for molecules that stabilize the high activity PAH multimer as an entry to small molecule therapies for PKU. AIM 3 addresses the allosteric regulation of a family of proteins, already identified as putatively using the morpheein model, whose dysregulation contributes to both IEMs and cancer. In all AIMS, we capitalize on novel applications of well established biochemical methods such as electrophoresis, chromatography, and enzyme activity determinations whose utility in the evaluation of protein quaternary structure dynamics is well established by our published work.

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Expected Research Outcomes and Benefits

The combined outcome of the project is an improved understanding of the quaternary structure dynamics of disease-associated human proteins. A unifying long-range goal is the identification of small molecules that can modulate protein quaternary structure dynamics for therapeutic advantage.

AIM 1 – We expect to identify octamer-stabilizing molecules that will provide 1) fundamental information on the role of quaternary structure dynamics to enzyme action and 2) potential therapies for patients with ALAD porphyria.
AIM 2 – We will unequivocally determine whether the allosteric regulation of PAH is dissociative; this defines the nature of the assemblies that can be targeted by small molecule PKU therapies. We will develop methods for high throughput screening of molecular libraries for compounds that can stabilize a high activity form of PAH. This will provide the tools necessary to define chemotypes that can be developed into pharmacological chaperones for treatment of PKU.

AIM 3 – We will define the quaternary structure equilibria associated with enzymes related to both IEM’s and cancer, opening avenues for the development of small molecule allosteric drugs to treat these diseases. We will develop methods to monitor the position of the equilibria of these proteins. The chosen targets are adenosylsuccinate lyase, geranylgeranyl pyrophosphate synthase, cystathionine-β-synthase, peroxiredoxin, pyruvate kinase, and dihydrolipoamide dehydrogenase. Any of these may prove intractable; however we expect to complete studies on at least three of these targets.

Summary of Research Completed

AIM 1 is directed at the identification of small molecules that will stabilize the active human PBGS octamer. We previously reported the details of a computational screen of the Life Chemicals, Inc. drug-like compound library and the identification of a total of 226 compounds to be used for the in vitro evaluation of compound effect on the PBGS quaternary structure equilibrium. Prior reported results with the designed PBGS variant R240A established the variant to be unsuitable for the screen.

During the current project period, we evaluated two disease-associated human PBGS variants, R240W and E89K, and found the latter to be most suitable for in vitro evaluation of the 226 candidate compound set; 109 compounds were newly acquired and 117 had initially been acquired for other projects and stored in dimethyl sulfoxide (DMSO) solution in our laboratory for 1–6 years. Each compound was incubated with human porphobilinogen synthase (PBGS) E89K at a protein concentration of 0.8 mg/ml and a compound concentration of 2 mM. Incubation was carried out for 2 h at 37 C and the resultant quaternary structure equilibrium position was determined using native polyacrylamide gel electrophoresis (PAGE) (12.5% acrylamide, PhastSystem). Three putative hit compounds were found to reproducibly stabilize the human PBGS octamer (Fig 1). These compounds are 7-[[4-(2-hydroxyethyl)-1-piperazinyl]phenyl-methyl]-8-quinolinol (coded as B6), 1-(benzo[d][1,3]dioxol-5-yl)-3-((4-morpholinophenyl)-amino)pyrrolidine-2,5-dione (coded as C9), and tetrahydro-5,10-diphenyl-1H,5H-Furo[3,4-c]furo[3′,4′:4,5]pyrazolo[1,2-a]pyrazole-1,3,6,8(3aH)-tetrone (coded as D4).

To test reproducibility, we identified the company TimTec as an alternate source for the three putative hits and have recently acquired the compounds from this source. Hit confirmation is ongoing.

AIM 2 broadly addresses control of the quaternary structure equilibrium of phenylalanine hydroxylase (PAH) as a therapeutic approach to phenylketonuria (PKU). One overriding goal of this aim is to obtain sufficient preliminary results to secure extramural (e.g. NIH) funding. For this reason, we have partially redirected our efforts to focus on structure; we have obtained both Small Angle X-ray Scattering (SAXS) profiles as well as the first X-ray crystal structure of a full
length mammalian PAH. Results for our initially designed experimental plan, as well as the new structural data are described herein.

Due to experimental challenges experienced during the first six months of this year (6/30/13-1/1/14), and those reported previously, the project had been modified to use heterologously expressed rat PAH in place of the originally proposed human protein (see AIM 2A below). The most significant challenge working with human PAH is its tendency to associate with other proteins during purification (e.g. stickiness) and the slow rate with which the protein relaxes from high-activity tetramer to the low-activity tetramer. This relaxation is essential for a well documented affinity purification using Phenyl Sepharose. These characteristics cause human PAH to elute from a Phenyl Sepharose affinity column both very slowly and bound to contaminating proteins, thus yielding a significantly less pure protein relative to rat PAH (as we had previously published). More recently we have improved our understanding of the PAH quaternary structure equilibrium and analyzed the interface between the regulatory and catalytic domains of the protein. Our model for PAH allostery predicts that this interface will dissolve during the transition from low-activity to high-activity tetrameric isoforms. Analysis of significant interdomain interactions along this interface revealed that position 29 is the only residue that differs between PAH from human (Cys29) and rat (Ser29). Therefore, we hypothesized that preparation of a C29S variant of human PAH may allow us to more effectively purify human PAH and to use this variant for studies most closely related to phenylketonuria. Indeed, we find that human PAH C29S purifies much like the wild type rat protein, in good yield, and is therefore a promising candidate for future studies. A recent expression and purification of this variant yielded 26 mg of >90% pure protein from the phenyl Sepharose affinity column and ~17 mg of homogeneous human PAH C29S after purification on a HiTrap Q-Sepharose column (gels illustrated in Figure 2). This is a dramatic improvement in both yield and purity over our published purification of wild type human PAH.

AIM 2A proposes to address the mechanism for interconversion between two PAH tetrameric isoforms, one high activity and one low activity. The high activity form is stabilized by binding phenylalanine at an allosteric site, rather than at the enzyme active site. A key aspect of this mechanistic question is whether the allosteric regulation of PAH requires multimer dissociation, as is characteristic of proteins that use the morpheein model of allostery. We had proposed to construct a His-tagged version of rat PAH along with specific designed variants that would allow us to utilize the subunit disproportionation method that we have described for the prototype morpheein. This method is challenging and requires preparation and characterization of several His-tagged rat PAH variants. During the current period, we have successfully constructed the plasmid and expressed and purified a His-tagged wild type rat PAH in high yield (24 mg affinity purified protein from 10.8 g cells); nickel affinity purification was carried out in the presence of phenylalanine to avoid isolating mixed isoforms. We find that the simple addition of a His-tag shifts the rat PAH equilibrium toward the high activity tetramer (~30%) relative to the native protein (~5%), which complicates the original experimental design. Plasmids for several designed variants of the His-tagged rat PAH have been prepared. However, prior to production and purification of these variants, we are exploring methods that simply monitor subunit exchange between tagged and untagged rat PAH under a variety of native conditions. This work is ongoing.
Our most significant results as to determining the mechanism of PAH allostery came from refocussing our efforts on using biophysical methods to probe the structure of full length wild-type rat PAH. We had previously demonstrated that two tetrameric isoforms of PAH are in slow exchange with each other and can be separated using ion exchange chromatography. A preparative separation was used to isolate PAH that is dramatically enriched in one tetrameric isoform (Fig 3a, Fraction 15). Our published work correlated this form with the low-activity tetramer. Fraction 15 was characterized using SAXS before and after addition of phenylalanine at a concentration sufficient to fully stabilize the high activity tetramer (Fig 3b), and was used to obtain the first ever crystal structure of full length mammalian PAH (Fig 3c).

The SAXS scattering data (Fig 3b, left) shows that a dramatic conformational change is stabilized by Phe. The observed scattering data qualitatively reflect the difference between SAXS scattering profiles predicted from our new X-ray crystal structure (reflecting the low-activity tetramer) and our published tetramer model for the high activity tetramer (Fig 3b, right). The latter includes a dramatic reorganization of the tetramer to form an interface between regulatory domains of adjacent subunits.

Multidomain multimeric allosteric proteins have historically proved refractory toward determination of their X-ray crystal structures, presumably because their intrinsic dynamics precludes formation of well-diffracting crystals. For this reason, all prior mammalian PAH structures have not been on the full length protein, but rather on truncated individual domains, or on two-domain constructs. Our ability to isolate an individual tetrameric isoform (Fraction 15) has now facilitated solution of a 3.7 Å crystal structure for the three-domain full-length protein. Although the low resolution and incomplete refinement precludes detailed discussion of molecular interactions, it is sufficient to conclude the following. First, there are distinct similarities with a composite homology model, first introduced by Ray Stevens and coworkers, for tetrameric PAH. These are: 1) the ACT domains of adjacent subunits are not interacting; 2) the auto-regulatory region is partially occluding the enzyme active site, consistent with that expected for the low-activity assembly; 3) the C-terminal multimerization domain is the primary region of inter-subunit contacts. The differences are: 1) the electron density in the C-terminal region does not fit the anti-parallel domain swapped 4-helix bundle seen in the PAH two-domain structure that first showed a PAH tetramer (PDB code 2PAH); 2) the orientation between adjacent active sites do not reflect that seen in the 2PAH tetramer. These differences support the hypothesis that the conformational change between the low-activity and the high-activity tetramer involve changes in overall protein architecture, consistent with (but not proving) a dissociative allosteric mechanism.

AIM 2B addresses the development of a high throughput in vitro method to identify compounds that can activate PAH by stabilizing the high activity tetramer. Significant effort was directed toward method development using a Wyatt Dynapro light scattering plate reader. Results using Phe (a known tetramer-stabilizing allosteric activator at concentrations about ~100 µM) and/or BH₄ (a known inhibitor) were found to be highly irreproducible. An alternate fluorescence-based method, where small molecules are screened for the ability to activate PAH activity remains viable. Using that method however, putative hits would need to be assayed for the ability to stabilize the PAH tetramer in a low throughput gel shift assay.
AIM 3 addresses the possible morpheein character of other proteins identified as drug targets for inborn errors of metabolism and/or cancer. We previously reported our progress on the protein adenylosuccinate lyase. No additional progress has been achieved toward this aim during the current reporting period.

**Figure 1**: Selection of 12.5% native PAGE screen of the 226 Life Chemicals Inc. compounds enriched for a propensity to stabilize the human PBGS octamer. In all gels, lane 1 is human PBGS variant E89K that had been incubated with carrier (DMSO); lane 2 is E89K in aqueous buffer; lanes 3 – 8 show E89K following incubation with various compounds dissolved in DMSO. Lanes with preliminary hit compounds are labelled with compound code. Structures of the preliminary hit compounds shown below the gels.

**Figure 2** – Human PAH variant C29S. Protein was expressed in E. coli and purified using the well established Phenyl Sepharose affinity method. The protein was then applied to a 1 ml HiTrap Q column and eluted with a salt gradient. These gels illustrate fractions through the peak containing PAH.
Figure 3 – New biophysical studies on full length rat PAH.  (a) Anion exchange (Hi Trap Q) chromatography is used to separate the two tetrameric PAH isoforms. Native PAGE, shown right, illustrates that Fraction 15 is predominantly one form. This fraction (at ~5 mg/ml) was used directly for SAXS and crystallographic studies. (b) Comparison of the experimental SAXS data (left) with the predicted scattering curves (right). Experimental scattering before (blue) and after (red) activation with Phe indicates a substantial difference in shape. Predicted SAXS scattering curves are shown for our new crystal structure (blue) and for the high activity protein structure model (red); this shows a qualitative similarity to the experimental data shown left. (c) Preliminary 3.7 Å crystal structure for the low-activity PAH tetramer derived from Fraction 15, in the absence of Phe. The N-terminal auto-regulatory region is pink; the ACT domain, which comprises the bulk of the regulatory domain is red; the catalytic domain is cyan; the multimerization domain is yellow. (d) Our previously published model for the high-activity mammalian PAH tetramer.
Research Project 3: Project Title and Purpose

Interactions of EGFR with the Scaffolding Protein NHERF1 and their Role in Cancer – The epidermal growth factor receptor (EGFR) plays a central role in controlling cell growth and differentiation of normal and malignant cells, and is an important target for cancer therapy. Efforts to develop improved cancer drugs depend critically on understanding how EGFR activity is controlled at the molecular level. This project is aimed at elucidating the role of a signaling adaptor protein, Na+/H+ exchanger regulatory factor 1 (NHERF), in controlling the activity and sub-cellular distribution of EGFR. Biophysical and cell-biological techniques will provide insight into the structural basis of NHERF-EGFR interactions and their impact on internalization and turnover of the cell-surface receptor.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

NHERF, a scaffold protein known to mediate the assembly of various signaling complexes at the membrane-cytoskeleton interface, has recently been identified as an important modulator of the activity and internalization of EGFR, an important oncogenic driver of many cancers. Cell-biological studies identified potential interaction sites in the C-terminal regulatory region of EGFR for the first PDZ (PSD95-Dlg1-Zo1) domain of NHERF. However, there have been no studies in vitro, and the structural details and biological implications of these interactions are poorly understood. To date, there have been conflicting reports on the possible roles of NHERF in cancer; depending on the cellular environment, NHERF can act as an oncogene or tumor suppressor. In this project we seek to fill these gaps in knowledge by combining biophysical with cell-biological approaches in order to (i) elucidate the structural basis of NHERF/EGFR interactions and (ii) to understand the impact of these interactions on receptor internalization and turnover. To accomplish the first aim, we will use nuclear magnetic resonance (NMR) spectroscopy to identify individual residues in each of the two PDZ domains of NHERF involved in binding synthetic peptides corresponding to likely binding motifs in EGFR. Heteronuclear 2D NMR spectra will be recorded on 15N-labeled NHERF samples at varying concentrations of unlabeled peptide ligands. To identify individual residues in EGFR that contact NHERF, we will measure the spectral perturbations in a recombinant 15N-labeled EGFR fragment caused by addition of unlabeled NHERF samples. The results, together with fluorescence-polarization measurements of binding affinity, will give detailed information on the structural and energetic basis of NHERF-EGFR interactions. To relate this insight to biology, we will investigate how NHERF modulates the trafficking of EGFR between the surface and interior of cells, using fluorescence microscopy and biochemical assays to observe receptor internalization induced by binding of fluorescence-tagged epidermal growth factor. Studies in the presence of EGFR inhibitors and mutant forms of NHERF or EGFR will give detailed insight into the mechanism by which NHERF regulates the sub-cellular localization and activity of EGFR.
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Expected Research Outcomes and Benefits

Molecules that block the growth-stimulating activity of EGFR are effective agents in clinical use for the treatment of several human cancers. However, some tumors do not respond to these drugs or develop resistance, and there is a continuing need for developing new and improved inhibitors of this highly oncogenic protein. These efforts will depend critically on understanding the mechanisms of EGFR activity at the molecular level. Binding of a growth factor to the extracellular domain of EGFR leads to self-association of the membrane-bound receptor and activation of the intracellular kinase domain; this in turn triggers a cascade of signaling events that are regulated by a network of cellular proteins. Another layer of complexity is added by ligand-induced internalization followed by degradation or recycling of the receptor to the plasma membrane, thus regulating the concentration of active EGFR on the cell surface. The signaling adaptor protein NHERF has recently been identified as an important modulator of this process. The proposed biophysical and cell-biological studies will allow us to elucidate the molecular basis of how NHERF recognizes EGFR, stabilizes it on the cell surface and participates in regulating its internalization. Since defects in EGFR receptor internalization have been linked with tumor aggressiveness and drug resistance, NHERF is a promising new target for cancer therapy. Ultimately, this project may lead to new predictive biomarkers for anti-EGFR agents and alternative approaches towards improving the efficacy of receptor-targeting therapies.

Summary of Research Completed

Preparation and characterization of NHERF and EGFR fragments. To identify the regions of NHERF involved in recognizing EGFR (Specific Aim 1), we prepared and characterized several fragments of this 358-residue scaffold protein, which are defined in Figure 1. The genes coding for these constructs, along with a cleavable N-terminal His$_6$ tag, were cloned into standard expression vectors (pET49b or pET151/D-top) and expressed in *E. coli*. As reported previously, we initially focused on the 1-97 fragment (PDZ1a), which spans the first PDZ domain as characterized in published X-ray crystallographic studies. However, this protein turned out to be relatively unstable. Thus, we prepared a longer fragment corresponding to residues 1-120 of NHERF (PDZ1b), which includes several conserved residues downstream from the canonical PDZ homology region. Circular dichroism (CD) spectra showed that the helix content of PDZ1b is approximately 2-fold higher than that of PDZ1a. This observation is consistent with a recently
published nuclear magnetic resonance (NMR) study that revealed a helical extension at the C-terminus of PDZ1. To determine the thermodynamic stability of PDZ1b, we recorded a series of CD and tyrosine fluorescence emission spectra as a function of urea concentration (PDZ1b does not contain tryptophan residues). Global analysis of the combined data, using methods developed previously by our group, showed that the unfolding transitions are well described by a cooperative (two-state) unfolding mechanism and yielded precise values for the unfolding free energy, \( \Delta G \). The \( \Delta G \) for PDZ1b (4.0 kcal/mol) is somewhat higher than that of PDZ1a (3.6 kcal/mol), indicating that the C-terminal helical extension contributes to stabilizing PDZ1. In addition to the individual PDZ domains, we also prepared a longer fragment, PDZ1-PDZ2 (1-263), which contains both PDZ domains along with a linker segment (~115-150). The linker contains few conserved residues and is predicted to be disordered. Finally, we recently optimized the conditions for expressing full-length NHERF at sufficient yields for biophysical experiments. This protein will also be used for Specific Aim 2. Figure 2 shows a schematic of the domain structure of EGFR, highlighting a segment of the cytoplasmic regulatory region containing two putative NHERF recognition motifs. Synthetic peptides corresponding to each of these motifs, EGFR-P1 and EGFR-P2, were obtained commercially. A 39-residue fragment spanning both motifs was expressed in *E. coli* as a cleavable glutathione S-transferase (GST) fusion. A longer recombinant construct comprising most of the C-terminal regulatory region of EGFR, EGFR-CT (966-1186) was obtained through collaboration with Mark Lemmon at the University of Pennsylvania.

**NMR resonance assignment.** To allow mapping of the binding interface for EGFR peptide ligands to individual residues of the NHERF PDZ domains, we first had to obtain backbone resonance assignments for PDZ1b and PDZ1-PDZ2 (assignments for PDZ2 were available from prior work supported by another grant). We prepared a uniformly \(^{13}\)C/\(^{15}\)N-labeled sample of PDZ1b by bacterial expression in minimal medium supplemented with \(^{13}\)C-glucose and \(^{15}\)N-ammonium sulfate. A series of two- and three-dimensional heteronuclear NMR spectra were recorded using standard pulse sequences, including \(^1\)H-\(^{15}\)N HSQC, HNCACB and HN(CO)CACB suitable for obtaining sequential backbone resonance assignments. Assigning the NMR spectrum of the 263-residue PDZ1-PDZ2 construct posed additional challenges due to its size and the presence of both ordered and disordered regions. The task was facilitated by the availability of resonance assignments for the individual PDZ domains. Interactions between the two domains appear to be relatively weak so that the spectrum of PDZ1-PDZ2 is well approximated by a superposition of the spectra of the isolated domains. Thus, we were able to obtain preliminary assignments for the majority of conformationally shifted peaks in the two-domain construct based on the assignments for the individual domains.

**Structural analysis of the interface between NHERF and EGFR ligand peptides (Specific Aim 1a).** To identify residues in PDZ1 involved in recognizing the first putative binding motif in the EGFR cytoplasmic tail, we recorded a series of 2D \(^1\)H-\(^{15}\)N HSQC spectra for PDZ1b (1 mM) in the presence of increasing concentrations of EGFR-P1 (0 to 10 mM). Many cross peaks experienced progressive ligand-induced chemical shift changes consistent with a dynamic binding equilibrium with a relatively short lifetime of the complex (≤10 ms). The data were analyzed by plotting the chemical shift perturbations (a weighted average between \(^1\)H and \(^{15}\)N shift changes) vs. ligand peptide concentration. A detailed analysis of the data showed that the different residues fall into two distinct groups. Group 1 residues showed chemical shift
perturbations in the high μM to low mM concentration range following simple binding curves, indicating formation of a 1:1 complex. Group 2 residues were unaffected by addition of ligand peptide up to several mM, followed by a steep sigmoidal binding curve at higher concentrations. By mapping the chemical shift perturbations onto the structure of PDZ1, we found that Group 1 residues are primarily located in and around a cleft between β-strand 2 and helix 2, which represents the binding site for canonical C-terminal PDZ ligand peptides. In contrast, Group 2 residues form a distinct cluster on the opposite face of the protein. Thus, EGFR-P1 can interact with two different sites on opposite faces of the PDZ1 domain. The sigmoidal shape of the binding curves for Group 2 residues indicates that the two binding sites are allosterically coupled, i.e., binding of a ligand to the first site triggers a conformational change resulting in increased affinity at the second site.

Towards defining the regions on EGFR-CT involved in contacts with NHERF. In an effort to identify the residues in the C-terminal tail of EGFR important for recognition of the PDZ-domains of NHERF, using NMR methods (Specific Aim 1b), we proposed to use an inverse labeling scheme, i.e., to complex 15N-labeled EGFR ligand peptides with unlabeled NHERF constructs. Since the cost of peptide synthesis with uniformly labeled amino acids is prohibitive, we chose a biosynthetic route to prepare a 39-residue peptide EGFR-P3 (Figure 2). A synthetic gene coding for residues 1033-1072 of EGFR fused to the C-terminus of GST, as well as a pair of His6 tags and tobacco etch virus (TEV) protease cleavage sites was obtained commercially and transfected into a suitable expression vector (pET49b). The fusion protein expresses well in E. coli, and affinity purification followed by proteolytic cleavage resulted in milligram quantities of pure EGFR-P3.

Fluorescence measurements of NHERF-EGFR binding affinity (Specific Aim 1c). To set the stage for fluorescence-based binding studies, we developed fluorescence polarization (FP) techniques for measuring binding affinities on a Perkin Elmer plate reader in 96-well format. FP is a sensitive and efficient technique for measuring the dissociation constant for a complex between a protein and fluorescence-labeled peptide ligands. The technique can be extended to unlabeled peptide or protein ligands if a fluorescence-tagged peptide is available that competes for the same binding site. The C-terminus of the cystic fibrosis transmembrane conductance regulator (CFTR) is a well characterized NHERF1 interaction partner that binds with sub-μM affinity to both of its PDZ domains. We purchased a synthetic peptide corresponding to the last ten residues at the C-terminus of CFTR with a fluorescein probe attached to its N-terminus (F*-CFTR10). FP measurements at a fixed concentration of F*-CFTR10 (100-200 nM) and variable concentrations of protein ligand (PDZ1b, PDZ1-PDZ2 or NHERF) exhibit a large increase in fluorescence polarization due to the decrease in the rotational correlation time of the fluorophore upon complex formation. These direct binding curves fit to a simple 1:1 binding model yielding dissociation constants of 85 ± 7 nM for PDZ1b, 840 ± 120 nM for PDZ2, 148 ± 17 nM for PDZ1-PDZ2 and 46 ± 8 nM for NHERF.

As a test for competition binding measurements, we titrated unlabeled CFTR10 into a mixture of protein with F*-CFTR10 under conditions where the labeled ligand is fully bound. We observed a sharp decrease in polarization with increasing concentration of CFTR as the unlabeled ligand displaces the labeled ligand. While such competition binding data are generally analyzed
empirically in terms of a simple 1:1 binding model (yielding a $K_i$), we developed a more rigorous approach by solving the equations for a three-state binding model, which makes it possible to quantify the dissociation constants for both the labeled and unlabeled ligand. In the case of the CFTR$_{10}$ complex with PDZ1-PDZ2, the Kd for the unlabeled form is substantially higher (490 ± 10 nM) than that for the labeled peptide (148 ± 17 nM), indicating that binding is enhanced by interactions between the fluoresceine group and the protein. Competition binding measurements avoid this potential pitfall by providing true $K_d$ values for the ligand in the absence of a fluorescence tag.

We used this competition method to monitor binding of the EGFR-P1 and EGFR-P2 ligands (Figure 2) to PDZ1b. Addition of EGFR-P2 to the preformed complex of PDZ1b with F*-CFTR$_{10}$ showed a marked decrease in FP at mM concentrations, indicating that this non-canonical ligand can displace a canonical C-terminal peptide ligand bound to PDZ1. The Kd of 1.5 ± 0.6 mM obtained is consistent with the binding curves for Group 1 residues observed by NMR. However, the corresponding experiment with EGFR-P1 failed to show any evidence for competitive binding up to the highest concentration studied (10 mM). Given our NMR evidence for complex formation described above, we conclude that EGFR-P1 is ineffective for displacing the CFTR ligand occupying the primary binding site, but can interact with the secondary binding site on the opposite face of the PDZ domain.

We also relied on our FP competition binding assay in order to detect interactions between the 221-residue EGFR-CT provided by our collaborators at the University of Pennsylvania with various NHERF constructs. When titrating the preformed complex between PDZ1-PDZ2 and F*-CFTR$_{10}$ with EGFR-CT, we observed a small, but significant increase in FP over the concentration range from 0 to ~100 µM, which fits to an apparent Kd of 17 µM. Since competitive binding would result in a large decrease in FP, this unexpected result is consistent with formation of a ternary complex. We hypothesize that two different internal sequence motifs in EGFR-CT, such as those highlighted in Figure 2, can recognize secondary binding sites on each of the tandem PDZ domains of NHERF without displacing the high-affinity C-terminal ligands bound to the primary binding cleft. The fact that the Kd for this interaction is two orders of magnitude lower than those for the separate binding motifs can be attributed to an avidity effect due to simultaneous recognition of two covalently linked binding sites.
Figure 1. Domain structure of NHERF1, including two tandem PDZ domains with C-terminal structural extensions, as well as a C-terminal ezrin binding motif (EB). Arrows indicate the recombinant fragments prepared for this project.

Figure 2. Domain structure of EGFR, focusing on the C-terminal regulatory region containing putative PDZ-recognition motifs (red). Arrows show the location of synthetic peptides and recombinant fragments prepared for this project.
Research Project 4: Project Title and Purpose

Epigenetic Reprogramming of Breast Cancer Metastasis – The incidence and mortality of triple negative breast cancer (TNBC) are increasing in women younger than 40 years old, who are more likely to be African American or Latina and who develop metastases in response to the conversion of epithelial to mesenchymal cancer cells (EMT). The purpose of this project is to use breast epithelial stem cells that are undergoing EMT for understanding first the epigenomic processes and second for developing preclinical studies targeting DNA methylation and histone deacetylation, processes that ensure transcriptional suppression, for implementing in this way a novel strategy for the treatment of TNBC.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

Numerous transcription factors (TFs) have been identified as drivers of oncogenic epithelial-mesenchymal-transition (EMT) and breast cancer metastases, representing a significant clinical problem since it promotes an aggressive stem cell phenotype, therapy resistance and tumor recurrence. The overarching goal of this project is to implement a novel strategic approach to treat metastases in triple negative breast cancer (TNBC) by targeting EMT. For this purpose we will aim first to unravel the epigenetic changes that drive the invasion, metastasis and maintenance of the cancer stem cells using MBD-Cap sequencing in an in vitro-in vivo model of EMT and cancer metastases developed in our laboratory. Secondly, we will develop a preclinical targeted therapeutic approach for reversing the epigenomic events in the EMT process as a treatment modality for controlling metastatic disease in TNBC. Therefore we are aiming at combining inhibition of multiple epigenetic targets that will likely be synergistic, as demonstrated for DNA methylation and histone deacetylation. We plan to test their single or combined effects on breast cancer metastasis, targeting the reversion of EMT using our in vitro-in vivo model. This approach is supported by the knowledge that cancer progression can be epigenetically controlled, as has been shown by the reversal of DNA hypermethylation and associated gene silencing that are accomplished through the use of two hypomethylating cytosine analogues that have been approved by the FDA for treatment of acute myelogenous leukemia and myelodysplastic syndrome. In addition, inhibition of EZH2, which involves the Polycomb Repressor Complexes (PCG) PRC1 and PRC2, is a potential target to be used for the abrogation of EMT by mediating gene silencing.

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Expected Research Outcomes and Benefits

Numerous outcomes and benefits are expected to emanate from this research project. The project aims at the abrogation of epithelial mesenchymal transition through the activation of novel epigenetic pathways as a mechanism for inhibiting the development of breast cancer metastases in minority populations who are at high risk of developing metastatic breast cancer at a young age. Results obtained through these studies will benefit young African American and Latina women who are diagnosed at an early stage (Phase I or II) with triple negative breast cancers that develop early metastases that result in elevated mortality. The identification of the epigenetic pathways through which epithelial mesenchymal transition drives the invasive and metastatic processes will generate essential knowledge that will contribute to solving the complexity of the problem under study and will benefit not only the population of women affected by triple negative breast cancer, but will also apply to the numerous types of cancer in which EMT drives the metastatic process. Understanding the pathogenesis of EMT in the breast cancer cells will provide novel tools for evaluating the response of metastatic disease to therapy. Our approach will allow us to identify novel therapeutic approaches such as the combination of epigenetic therapy with histone methyltransferase inhibitors and histone deacetylase inhibitors for the abrogation of EMT that ultimately will inhibit the development of metastases. The knowledge we will gain on the epigenetic reversion of EMT as a tool for preventing the development of metastasis will allow us to use these inhibitors in clinical trials in breast cancer.

Summary of Research Completed

**Aim 1**

To understand the methylation changes throughout the EMT process, we have performed MBDCap sequencing of the cell lines of our EMT *in vitro* model (MCF-10F, trMCF and bsMCF) and bsMCF cell treated with 500 nM SGI-110 for four days. SGI-110 was selected based on the results obtained in our *in vitro* studies (Aim 2). Each cell line had DNA extracted from 4 consecutives passages, totaling 16 samples. DNA was then fragmented to 150-200bp sizes using Covaris® S2 system (Covaris, Inc.). 2µg of fragmented DNA were used for Methylated DNA enrichment using MethylMiner™ Methylated DNA Enrichment Kit (Life Technologies) following the manufacturer’s protocol. The MBDCap technique makes use of the methyl-CpG binding domain of human MBD2 protein, which is coupled with magnetic beads. The MBD has affinity for methylated cytosine residues. The captured methylated DNA was eluted using a high concentration (2M) of NaCl. Approximately 1-2% of methylated-DNA was obtained from the input. The samples were then sent to the Genome Sequencing Facility in the Greehey Children's Cancer Research Institute at the University of Texas Health Science Center at San Antonio for library preparation, sequencing and bioinformatics analyses. Library preparation was performed in the Beckman-Coulter SpriWorks automated library prep. Library fragments sizes ranged from 290-330bp and concentration 7.64-34.9nM. HiSeq2000 (Illumina® Inc) was used for sequencing of 4 samples/lane, 50 bp single reads. The samples have been
successfully sequenced and analyses are underway.

**Aim 2**

In previous reports, we have shown that we examined the effects of two DNA methyltransferase (DNMT) inhibitors, DAC and SGI-110 (SGI), and six histone deacetylase (HDAC) inhibitors, Vorinostat (SAHA), JNJ-26481585 (JNJ), Entinostat (MS275), SB939, LBH589, and Tubastatin A HCL (Tub) on the cell growth, motility, and invasion. The effects on three dimensional cultures in collagen matrix and colony formation were measured with the treatment of DAC, SGI, JNJ, and SAHA only. Our results suggested the new generation DNMT inhibitor SGI is superior to the previous DNMT inhibitor DAC. To determine which HDAC inhibitor is the best one to reverse EMT process, we performed a collagen assay and an agar methocel assay with the four HDAC inhibitors (MS275, SB939, LBH589, and Tub) which were tested by MTT assay and showed great inhibition in cell growth. Then we treated cells with SGI in combination with selected HDAC inhibitors and measured the effects on EMT process. Cell cycle analysis and western blotting were performed to unveil the possible mechanisms. The TNBC mouse models were established and *in vivo* study was carried out by treating mice with SGI. The data presented below show the milestones we achieved for this period in aim 2.

**Changes in cell morphology**

LBH and Tub induced more cells in a large size and longer dendrites than that induced by DAC and SGI. SB939 treated cells were similar to JNJ treated except that the cells were flattened after SB939 treatment. Differently, all cells treated with MS275 were enlarged greatly in size and had a flat appearance.

**Collagen assay for determining ductulogenesis/solid masses formation assay**

To determine how the compounds treatment affects cell behavior in a three dimensional collagen matrix, collagen assay was performed. Treatment of all four compounds increases the number of masses. The MS275 treated group showed more masses with smooth outer surfaces, and less disseminated growth of individual cells, indicating that the HDAC inhibitors are able to reverse mesenchymal-like growth in this environment; MS275 is the best one among these four compounds.

**Agar Methocel Assay**

The colony formation ability of the cells was measured by agar methocel assay. Treatment of MS275, SB939, and Tub reduced the number of colonies compared to the control. MS275 and SB939 are better than Tub in the reduction of colony numbers (Figure 1A-1B).

**Combined treatment of DNMT inhibitors with HDAC inhibitors**

The treatment of DNMT inhibitors in combination with HDAC inhibitors has shown promising results in some studies. We selected the best compound tested in bsMCF and did combination treatment. Cells were treated with SGI, SAHA, MS275, or SGI combined with SAHA, SGI combined with MS275 for 96 hours and used for MTT assay, wound healing assay, collagen assay, and agar methocel assay. Combination of SGI (500 nM) with SAHA (700 nM) or MS275 (1000 nM) could induce greater inhibition in cell proliferation and migration of the bsMCF cell line (Figure 2A-2B). In collagen assay, there was no significant difference between the single compound treatment and combined treatment in the number of masses. The classification of
masses showed SGI combined with SAHA of MS275 increased the number of masses with smooth outer surfaces more than SAHA or MS275 treatment alone. Agar methocel assay also showed that SGI combined with SAHA of MS275 induced greater reduction in the formation of colonies compared to SAHA or MS275 treatment alone. The colony numbers were 1267±278 for the control, 223±63 for SGI treated, 458±76 for SAHA treated, 550±40 for MS275 treated, 402±38 for SGI+SAHA treated, and 325±50 for SGI+MS275 treated.

Cell cycle analysis
To analyze the treatment of compounds on cell cycle, we conducted flow cytometry analysis. Cells were treated in the flasks for 96 hours and used for analysis. Upon treatment with MS275, or combined treatment of SGI with SAHA or MS275, a strong G1 phase arrest was observed in the bsMCF cell line (60.8% for control, 71.4% for MS275, 78.0% for SGI+SAHA, and 81.0% for SGI+MS275).

Western blot analysis
To investigate whether the treatment results in changes in the expression of EMT markers, cells were plated in 10 cm dishes and treated with compounds for 96 hours. At the end of treatment, cell lysates were made using radio immune precipitation (RIPA) buffer. Protein expression was examined by western blot. All treatments induced up-regulation of E-cadherin in bsMCF cells. Combined treatment of SGI with MS275 reduced the expression of both vimentin and slug in bsMCF cells as well (Figure 2C).

Development of TNBC mouse models for in vivo study
Although the bsMCF cell line is tumorigenic and metastatic in SCID mice, to form lung metastasis, we must inject over 2,000,000 cells per mouse, which is lethal for many mice. We developed two additional cell lines from bsMCF cells (Figure 3). The XtMCF cell line was derived from xenograft tumors of bsMCF cells, and the LmMCF cell line was derived from lung metastasis of bsMCF cells. Both of the two cell lines are negative for E-cadherin and highly positive for vimentin by immunofluorescent staining and are tumorigenic and metastatic in SCID mice. The xenograft tumors can reach a size of 9 mm in diameter four weeks after injection with 2,000,000 cells. For the lung metastatic model, tail vein injection of 1,000,000 cells per mouse results in the formation of lung metastases in 100% of mice. Mice start to die three weeks after cells injection. The lung metastases in the LmMCF model were much more severe than in the XtMCF model. The immunohistochemical staining of the estrogen receptor alpha (ER), progesterone receptor (PR), and human epidermal growth receptor 2 (HER2) confirm that XtMCF and LmMCF are TNBC cell lines (Figure 4).

To test if we can use SGI to treat XtMCF and LmMCF cell lines for in vivo study, MTT assay was performed to test the sensitivity of these two cell lines to SGI treatment. Both the XtMCF and LmMCF cell lines are sensitive to the treatment of SGI in vitro. The IC50 is 230±14 nM for XtMCF, and 378±52 nM for LmMCF.

In vivo anti-metastasis activity
XtMCF and LmMCF cells grown in vitro were suspended in PBS and 600,000 XtMCF cells or 500,000 LmMCF cells were injected into tail vein of 7 week old CB17/SCID mice. Mice were allowed to rest for two days; treatment was initiated on the third day following cell injection,
with a schedule of Monday, Wednesday, Friday subcutaneous injection. The dosage of SGI was 2.0 mg/kg for the XtMCF model, and 1.5 mg/kg, 2.0 mg/kg for the LmMCF model.

After two weeks of treatment, in the XtMCF cell line model, five out of six mice lost 3.7% to 26.3% of body weight. In the LmMCF cell line model, mice treated with 2.0 mg/kg SGI lost 8.2% to 13.6% of body weight; in contrast, four out of five mice treated with 1.5 mg/kg (7 doses) gained 2.5% to 7.8% of body weight. Mice were treated for two weeks and then sacrificed, and lungs, livers, hearts, and brains were collected, stained with Bouin’s solution, and examined. In the LmMCF cell line model, all lungs showed extensive metastases on the lung surface. It was impossible to count them as a lot of metastases fused already. Comparing lungs of SGI treated mice to that of the control, the SGI group had a little bit of normal-like lung tissue between metastases; however, it was hard to find normal-like lung surface in the control lungs.

To examine the severity of the metastasis in the lungs of the LmMCF cell line model, paraffin sections were stained with hematoxylin and eosin (H&E). All the lungs from control mice showed a thick layer of tumor cells on the lung surface and a lot of metastatic nodules inside the lung. However, the majority of lungs from SGI-treated mice showed a thin layer of tumor cells on the lung surface. The metastasis in the 1.5 mg/kg SGI group was not as severe as in the 2.0 mg/kg SGI group. The severest lung metastases of each group are shown in Figure 5A. The presence of tumor cells in the lung is confirmed by the immunostaining with antibody to human vimentin (Figure 5B).

The schedule of 2.0 mg/kg SGI, three times a week may not be the best schedule for the XtMCF cell model, since the mice lost too much body weight after treatment, and the examination of lungs by H&E staining and immunostaining of vimentin didn’t show a significant difference from the control.

As the XtMCF and LmMCF cell lines are highly tumorigenic and metastatic in CB17/SCID mice, the combination treatment for in vivo studies is considered. We tested the sensitivity of XtMCF and LmMCF to the treatment of SAHA and MS275 and the combination of SGI with SAHA or MS275 in vitro by MTT assay. The sensitivity of the XtMCF and LmMCF cell lines to SAHA are similar to that of the bsMCF cell line (IC₅₀ is 680 nM for XtMCF, 690 nM for LmMCF, and 715 nM for bsMCF). In contrast, both the XtMCF and LmMCF cell lines are more sensitive to the treatment of MS275 compared to the parent cell line bsMCF (IC₅₀ is 1,000 nM for bsMCF, 717 nM for XtMCF, and 265 nM for LmMCF). The combination of SGI with SAHA or MS275 induces greater cell growth inhibition compared to one compound treatment alone (Figure 6). The in vivo studies (xenograft and metastatic model) of these two cell lines with the treatment of SGI, MS275, and SGI combined with SGI are underway.
**Figure 1**: Agar methocel assay shows MS275, SB939, and Tub reduce the number of colonies. * indicates p<0.05 compared to control. ** indicates p<0.01 compared to control.

**Figure 2**: Combination treatment of SGI with MS275 induces greater inhibition in cell growth (A), motility (B), up-regulates E-cadherin and down-regulates vimentin (C). * indicates p<0.05 compared to control. ** indicates p<0.01 compared to control. † indicates P<0.01 when comparing SGI+SAHA with SAHA, or SGI+MS275 with MS275.
Figure 3: *In-vitro-in-vivo* cell model. XtMCF and LmMCF cell line were developed from xenograft tumor or lung metastasis of bsMCF cells, respectively.

Figure 4: Immunohistochemical staining of xenograft tumors and lung metastases from XtMCF and LmMCF cell lines. The staining results confirm that XtMCF and LmMCF cell lines are triple negative breast cancer cell lines.
Figure 5: The treatment of SGI reduces tumor burden in the lungs. CB17/SCID mice were injected with 500,000 LmMCF cells into tail vein. Treatment was initiated 3 days after injection. Mice were sacrificed 18 days after cells injection. Histopathological examination by H&E staining is shown in A. Immunohistochemical staining of lungs with antibody to human vimentin is shown in B.

Figure 6: The growth of XtMCF and LmMCF cell lines is sensitive to the MS275 treatment. Combination of SGI with SAHA or MS275 induces greater inhibition than single compound treatment alone. ** indicates p<0.01 compared to control. † indicates P<0.01 when comparing combined treatment to one compound treatment alone.

Research Project 5: Project Title and Purpose

New Approach to Identifying Poly(ADP-ribose)Polymerase 1 Inhibitors – The purpose of this research project is to develop and test a new class of Poly(ADP-ribose) Polymerase 1 (PARP-1) inhibitors for targeting and eliminating cancer cells. Our new assay is based on the protein-dependent pathway of PARP-1 activation. It will be used to screen for inhibitors, as well as to determine their mechanism of action. This method allows determining whether the inhibitors impair PARP-1 by targeting gene transcription or DNA repair pathway, thus aiding the in discovery of new drugs of superior specificity to be used in future treatment protocols. Our method will identify novel classes of PARP-1 inhibitors that disrupt PARP-1 activation by targeting its interaction with specific proteins.

Duration of Project

1/1/2013 – 6/30/2014
Project Overview

During the past few years, Poly(ADP-ribose)Polymerase 1 (PARP-1) proteins have become a very popular target for anti-cancer treatment. Many PARP-1 inhibitors have been generated and tested by the pharmacological industry. However, most of these were designed to disrupt the DNA-dependent PARP-1 protein activation pathway, based on competition with NAD for a binding site on the PARP molecule. Therefore, their action also depended on disruption of the PARP-1 mediated enzymatic reaction. This limitation resulted mainly in the discovery of nucleotide-like PARP-1-inhibitors that may target not only PARPs, but also other enzymatic pathways involving NAD and nucleotides as co-factors. Here, we suggest a strategy for the identification of PARP inhibitors that target a different pathway, histone H4 dependent PARP-1 activation. In addition to identification of NAD competitors in a small molecules collection, this approach allows the discovery of novel classes of PARP inhibitors that only disrupt H4 based steps of PARP-1 activation and therefore will be more specific and more effective.

Aim 1. Small-molecule collection and high-throughput colorimetric assay to identify PARP-1 inhibitors. Here we develop and perform an assay suitable for assessing PARP-1 inhibition by a subset of 90,000 drugs within the Institute for Cancer Research, Fox Chase Cancer Center Translational Research Library.

Aim 2. Explore the potentiality of H2A, H2Av, and H4 N-terminal tails as PARP inhibitors in vivo; develop novel therapeutics. Our past in vitro work on PARP activation has demonstrated that the N-terminal tails of H2A and H2Av inhibit PARP, whereas the N-terminal tail of H4 works to activate it. With research on and the development of peptide drugs emerging rapidly in the pharmaceutical industry, it has been suggested that these short (approximately 30 amino acid) peptides can act as future PARP inhibitors for treating cancer. Transgenic flies are currently being designed to overexpress these peptide inhibitors in order to understand the potential for inhibition in vivo. The second step would include purifying batches of the peptide inhibitors and testing their effectiveness in killing BRCA-1/BRCA-2 deficient tumor cells.

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Expected Research Outcomes and Benefits

Our project targets cancers that are sensitive to drugs that reduce the ability of cancer cells to repair their DNA. Such cancers affect large numbers of Pennsylvania residents, particularly
breast and prostate cancers (10,255 and 10,098 cases in 2008 respectively). Following research indicating that PARP-1 inhibitors have anti-tumor activity in breast, ovarian, and prostate cancers associated with BRCA1/2 mutations, PARP-1 proteins have become a very popular target for anti-cancer treatment. The pharmacological industry has generated and tested many PARP-1 inhibitors. However, several setbacks in clinical research on PARP-1 inhibitors indicate that current strategies used to design inhibitors lead to discovery of molecules that have low specificity to PARP-1 and/or affect other pathways as well. In this project, we develop a new strategy for identifying PARP-1 inhibitors that will ensure high specificity and effectiveness of newly identified molecules in eliminating cancer cells. We expect that a novel class of inhibitors will find immediate use in clinical applications and will be effective not only in combination with chemotherapy or irradiation, but also in monotherapy. Therefore, this novel strategy will improve anti-tumor treatments and will result in lower mortality rates and longer post-treatment life-expectancy among patients. Furthermore, since our strategy will produce drugs with superior specificity against tumorigenic cells, we anticipate improved quality of life during/after treatment due to lower levels of side effects, as well as decreased cost of therapy. Moreover, by testing multiple classes of small molecules – PARP-1 inhibitors, we expect to be able to identify small molecules effective for specific tumor types thereby laying the foundation for “personalized medicine.”

**Summary of Research Completed**

**RCC malignancy is coupled with aberrations in pADPr turnover.** Poly(ADP-ribose) (pADPr) is overaccumulated in a variety of cancers. Its accumulation has been associated with poor prognosis for cancer patients. We have examined pADPr levels in established and primary patient-derived malignant cell lines. The most severe misregulation of pADPr turnover was found in renal cell carcinoma (RCC) cell lines expressing an unusually high level of the PARP-1 protein. In contrast, expression levels of PARP-1 were significantly lower in normal kidney cells. pADPr turnover is regulated by the enzyme poly(ADP-ribose) glycohydrolase (PARG), which degrades it to free ADP-ribose and adenosine monophosphate (AMP). Notably, PARG expression is significantly higher in normal cells than in malignant RCC cells. Expression of E-Cadherin, which we use as a biomarker of PARP-1 pathway disruption, is also reduced. Thus, RCC cells represent an optimal model for studying pharmacological potential of PARP-1 activity in limiting cancer progression.

**PARP-1 controls expression of a large subset of genes involved in the regulation of cell identity and malignant transformation.** Activation of PARP-1 in chromatin in response to developmental and environmental stress mediates chromatin decondensation and stimulates transcription. PARP-1 is unevenly distributed throughout chromatin and localizes in promoter regions of specific genes. This makes PARP-1 a plausible epigenetic memory factor, capable of marking specific loci and regulating their expression during normal development and pathogenic transformation. We used ChIP-seq to examine genome-wide PARP-1 localization in human embryonic kidney cells. We found that PARP-1 localizes inside 7242 loci and controls their expression. Most identified PARP-1-dependent genes are involved in cellular adhesion or intercellular interaction, and, hence, responsible for maintaining cell identity. Since cells with misregulation of these functions demonstrate high risk of malignant transformation and display a tendency to metastasize, labeling of these genes by PARP-1 restores cellular identity required for
a cell-cell niche interaction, thus preventing uncontrolled cell migration and potentially tumorigenic dedifferentiation. Virtually all genes involved cancerogenesis reported by National Institutes of Health (NIH) resources were identified as PARP-1 targets by our chip-seq screen.

In the human genome, PARP-1 controlled genes included all factors/proto-oncogenes commonly expressed in cancer cells, including Myc, TMPRSS2, Insulin growth factor, Her-2, EGFR, Wnt, MMPs, and --most relevant for this proposal-- VHL and HIF, which are involved in RCC malignancy. We observed that elevated expression of Myc, typical for cancer-derived cells, could be suppressed by culturing these cells with moderate amounts of PARP-1 inhibitors. Taken together, these findings suggest that PARP-1 may regulate malignancy of RCC by controlling transcription of cancer-specific loci.

**Novel non-NAD-like PARP-1 inhibitors effectively suppress growth of RCC cells, both in vitro and in vivo.** PARP-1 is a NAD-dependent enzyme, activity of which can be suppressed by introducing NAD competitors, as well as by two additional routes: obstruction of PARP-1 binding with DNA and disruption of PARP-1 interaction with histones. The majority of currently known PARP-1 inhibitors have been designed by targeting the first route, as NAD-mimetics. In this study, we used four classical NAD-like inhibitors for control and comparative purposes: 3AB, 4-ANI, PJ34, and Olaparib. Using our knowledge about the molecular mechanisms underlying PARP-1 regulation, we developed a novel strategy for designing PARP-1 inhibitors, which allows to regulate PARP-1 activity with great precision and specificity.

Using histone H4-dependent PARP-1 activation, we developed and performed a high-throughput screen to identify PARP-1 inhibitors. We identified 856 new PARP-1 inhibitors and tested a selected subset of 156 inhibitors for their ability to eliminate cancer cells selectively. Treatment of cancer cells with inhibitors discovered during the screen demonstrated the superior efficacy of these novel inhibitors in blocking PARP-1 activity in vivo, as compared to classical PARP-1 inhibitors PJ34 and 4-ANI. These new inhibitors eliminated cancer cells (breast, prostate, and lymphomas) with higher specificity than classical ones. We have screened small molecules and selected those compounds that inhibit only histone-dependent PARP-1 activation. Our data demonstrate a superior selectivity of new histone-dependent PARP-1 inhibitors toward blocking activation of this enzyme in vitro. These new inhibitors suppress cancer cells with higher specificity than the classical PARP-1 inhibitor Olaparib and synergistically interact with it during dual treatment. In light of encouraging in vitro data, we next examined the antitumor activity of non-NAD-like inhibitors using RCC xenograft tumors established from patient-derived tumor cells.

**Research Project 6: Project Title and Purpose**

**Cell Culture Facility Research Infrastructure Renovation** – The purpose of this project is to renovate existing space that will accommodate laboratory and office space for the Cell Culture Facility (CCF). The CCF supports the culture of mammalian cells by research laboratories at The Institute for Cancer Research (ICR), Fox Chase Cancer Center (FCCC) by providing culture media, supplies, technical expertise, and specialized equipment. Currently, the CCF research space, equipment, and offices are decentralized across four distant rooms and two hallways on three floors of two adjoining buildings. The renovation will modernize the central laboratory...
and adjacent laboratory space to consolidate all components of the facility into a centralized location and greatly improve the capacity of the facility to provide state-of-the-art cell culture services.

**Duration of Project**

1/1/2013 – 6/30/2014

**Project Overview**

The purpose of this project is to renovate and centralize the operations of the Cell Culture Facility at Fox Chase Cancer Center, which provides cell culture research support services to most laboratories within the Center. Currently, the facility operations are scattered across four physical spaces on three floors of two adjoining buildings and physical conditions of the laboratory space is inefficiently designed and in dire need of renovation to provide optimal sterile cell culture support services within a centralized space. Existing space encompassing the current central laboratory, an adjacent hallway, and another adjacent laboratory/office will be renovated to accommodate the entire facility in one location. The renovation project will be performed in phases to move operations into the adjacent laboratory while the central lab, hallway, and office space are renovated, followed by relocation of operations to the renovated central lab and subsequent renovation of the adjacent lab space to house the culture media preparation operations, which is currently located in the basement of the adjoining building. Upon completion of the renovation, the renovated facility will be housed in a central location on a single floor in state-of-the-art laboratory space, which will significantly improve capacity to provide cell culture support services to all research laboratories within the entire institution.

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**Other Participating Researchers**

Pamela Nakajima, PhD – employed by The Institute for Cancer Research

**Expected Research Outcomes and Benefits**

The renovation of the Cell Culture Facility (CCF) will provide significantly improved centrally located physical space that will allow for improved operations of all facility activities. The CCF is one of 16 core research facilities at the Institute for Cancer Research, Fox Chase Cancer Center that provide a variety of specialized support services for all research laboratories. Cell culture technology plays an essential role in modern cancer research. The CCF serves as a customized pay-for-service facility that provides expertise, technical support, reagents,
equipment, and training to increase the effectiveness and efficiency of using mammalian cell
culture in the laboratories. The CCF provides technical and consultation services in tissue culture
techniques, hybridoma production, centralized liquid nitrogen banking, mycoplasma screening
services, the preparation of custom and standard culture media and supplements, performance
testing of fetal bovine serum, cell propagation, and technical support and supplies for mouse
embryonic stem (ES) cell transfection and culture. Importantly, the CCF supplies the ICR/FCCC
community with valuable on-site expertise for a host of established cell culture protocols,
consultation in the design and evaluation of experiments, training in cell culture methodologies,
high quality culture media and supplements, and adaptable assistance delivered in a cost-
effective manner. Although the CCF does not contribute directly to improvements of health
status, it allows individual laboratories to focus funds and laboratory activities on direct research
needs by providing increased efficiency in obtaining materials, specialized technical cell culture
services using established protocols, on-site expertise, and equipment. Ultimately, improved
research efficiencies lead to experimental results that improve human health.

Summary of Research Completed

The CCF renovation was recently completed, resulting in an outstanding environment with
substantially improved infrastructure and workplace efficiency. The renovation fulfilled all three
of the originally proposed specific aims:

Aim 1. Renovate current CCF main laboratory space and install new filtered ventilation

In total, the entire interior of the original lab footprint was updated, and a hallway and another
adjacent laboratory space were renovated and incorporated into the final floor plan. The
renovation was primarily performed by FCCC Maintenance Department staff, which lengthened
the construction time, but allowed for greater coordination of the work schedule and substantial
cost savings.

The original lab (Reimann 288) was completely stripped of floor tiles, ceiling and cabinetry, and
floor plan was reconfigured to be more efficient. Walls were repainted, new flooring, lighting,
and ceiling tiles were installed, and new electrical outlets, plumbing, Ethernet, vacuum lines, and
gas lines were installed. A large island in the middle of the original lab was always awkward,
and this was removed and replaced by two smaller islands with more useful countertop space,
drawers, cabinets, and shelves. One island has a centrally located sink. The reconfiguration of
space allowed us to better configure the four biosafety hoods in the main lab along walls,
whereas in the original lab, they were clustered at one end of the lab, resulting in congestion of
personnel. A desk space was installed next to each biosafety hood workstation and PC
computers were purchased and installed with wiring to the FCCC intranet and internet, thereby
allowing for local digital documentation of work. In addition, chairs and compact stainless steel
rolling carts with a drawer and shelves were purchased to provide optimal workstations for all
technical staff.

In addition, new ventilation ductwork was installed throughout the lab. A contractor was called
in to safely remove asbestos wrapping found while renovating the ventilation. Importantly, air
filters were installed on all ceiling ventilation ducts to improve air quality, which is critical for a
modern cell culture laboratory.

Aim 2. Incorporate adjacent hallway and lab space to consolidate services in one centralized location

A long hallway that originally led to the main laboratory and other rooms of the facility was fitted with a large door outside of Reimann (R) 284 to incorporate this hallway into the facility. This door is opened during regular business hours on weekdays and was fitted with an automatic device to close at 6 PM. The door can be opened by FCCC research personnel with standard identification badges for ready access at all hours to obtain cell culture supplies or use equipment. This provides security and capacity to monitor who has entered when the facility is closed. The new doorway thereby secures access to R284, R288 (main lab), and the R289 suite of rooms, including the walk-in cold room.

R284 was stripped of desks and sink to create a central liquid nitrogen banking repository. This now includes three very large liquid nitrogen tanks that each have capacity to hold 20,000 vials. One of these tanks was purchased with funds from the CURE grant, whereas the other two had been purchased using institutional funds in recent years. In addition, another large liquid CCF nitrogen tank was moved to R284 from a satellite room in a distant portion of the Reimann Building. Now the facility is fully populated with state-of-the-art liquid nitrogen tanks linked to automatic filling regulators and alarms to accommodate all banking needs for labs in the entire institute. Location and contents of all of the vials within these tanks (over 50,000) are fully detailed in a computer database. In addition, these tanks are in a central location and numerous smaller tanks have been retired to improve efficiency, and our volume of liquid nitrogen usage has decreased dramatically. Furthermore, a nitrogen alarm was installed in the room to protect personnel in the case of excessive nitrogen buildup.

The R289 suite has been converted to multiuse space, some of which was not previously part of the CCF. R289B was modified to house common use equipment for ready access to FCCC researchers, even when the facility is closed. This equipment includes an Amaxa Nucleofector, ACEA xCELLigence cell growth monitor, low oxygen incubator, immunofluorescence microscope with a digital camera, and a biosafety hood. Another space across the hall from 289C was lined with shelves to use for storage. In addition, R289B was renovated as an office space for the CCF Manager, Dr. Pamela Nakajima.

In addition, the adjacent laboratory, R289, was incorporated into the facility to house media preparation operations. This space was previously a lab for the laboratory animal facility, and was fitted with new flooring, new ceiling tiles, paint, and cabinet modifications to create an efficient workspace. A sophisticated Millipore water purification system was also moved into this lab space to provide high quality water for media preparation. The media preparation kitchen was previously located in a distant location (R151), so moving to this adjacent location further centralized the facility operations.

Aim 3. Continue to provide full CCF services to the FCCC research community during the renovation process

A carefully coordinated schedule of construction was arranged during the renovation period.

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This involved rotating the staff into temporary quarters in an adjacent laboratory (R290) while the main lab was modified, and then moving the staff into the renovated laboratory, while R290 was renovated and converted into the media preparation laboratory. In addition, several GEM box refrigeration units and freezers were positioned outside of R290 to make media preparations available to FCCC staff during the early phase when the cold room was inaccessible. Therefore, while the staff was housed in close quarters for several months, the facility was fully operational and capable of providing all services during the entire renovation period. The move of the media preparation kitchen to R290 was the final step in the renovation, and this move from R151 only disrupted operations for a couple of days when the Millipore water purification units were moved. In addition, all biosafety hoods were recertified after moving to R290 and again after moving back to R288, thereby preventing any contamination conditions and assuring biosafety of lab personnel.

*Equipment Purchases*

Since the majority of the renovation work was performed by internal staff from the FCCC Maintenance Department, we were able to purchase several valuable pieces of equipment to further enhance capabilities of the CCF. In addition to the liquid nitrogen tank and five small computers previously mentioned, funds were used to purchase a -80°C freezer, small centrifuge, Nexcelom Bioscience Cellometer K2 cell counter (to replace an old Coulter Counter), Nikon digital camera for a fluorescence microscope, a refrigerator for the media preparation kitchen, small CO2 incubator, stereomicroscope for tissue dissection, and large GEM Box refrigerator. In addition, new chairs and filing cabinets were purchased. All of these purchases were carefully considered to provide maximal benefit to improve facility services, and much of the equipment is available for use by FCCC researchers.