National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation

Annual Progress Report: 2012 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

The NSABP Foundation received $967,037 in formula funds for the grant award period January 1, 2013 through June 30, 2016. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Evaluation and Mechanistic Studies of Molecularly Targeted Agents in Colon Cancer – The purpose of this project is to evaluate the efficacy of molecularly targeted agents, alone and in combination, on cell viability. Signaling pathway studies will be conducted to understand mechanisms of resistance and sensitivity to specific agents. Molecular profiling studies will be conducted with the purpose of identifying molecular subtypes in colon cancer cell lines that have been shown to be particularly resistant to therapy and identifying potential combinations of targeted therapies that may overcome this resistance. Identification of these agents and the mechanisms responsible for their success and failure in our studies will lead to new and improved approaches to colon cancer treatment..

Anticipated Duration of Project

1/1/2013 – 6/30/2016

Project Overview

The broad research objectives are to provide meaningful pre-clinical data as a basis for developing clinical trials. Therefore, we propose to identify new therapeutic agents and agent combinations for treatment of colon cancer patients. To do this, we propose these specific aims:
AIM 1- Identify new agents and agent combinations to optimize colon cancer cell killing;
AIM 2 - Determine the mechanisms responsible for drug resistance and sensitivity of commonly used targeted therapies and newly discovered drug combinations that have been shown to be particularly effective in killing tumor cells;
AIM 3 - Identify new druggable targets in treatment-refractory colon cancer cell lines.

For the first aim, we will test targeted agents and agent combinations for their effect on a defined set of colon cancer cells to identify new agents and new agent combinations that affect cell viability using a high-throughput screening strategy. Cell lines and primary colon cancer cells

from patients with limited passage history will be used. Molecular profiling data from these studies and from the literature will be used to develop hypotheses about why these primary cancer cells and cell lines have resistance or sensitivity to specific agents. Molecular profiling of primary cells will be carried out, but for most of the cell lines, molecular profiling data (copy number, sequence data, whole transcriptome analysis) are already available.

Aim 2 will be carried out using cell lines that are resistant and sensitive to targeted agents. Phosphorylation studies will be done with multiplex assays and Luminex xMAP technology.

The purpose of aim 3 is to identify new targets using a cell line that is resistant to a large number of agents. One or two resistant cell lines will be selected from information on cell lines available from published data and from data gathered in aim 1. New potential druggable targets will be identified using a short hairpin RNA (shRNA) screen. Differential representation of shRNAs in the cell lines grown in the presence or absence of chemotherapy will be identified by Next Generation Sequencing analysis. Each shRNA in the library can be identified by Polymerase Chain Reaction (PCR) amplification of unique barcode identifiers.

Principal Investigator

Katherine Pogue-Geile, PhD
Assistant Director Molecular Profiling
NSABP Foundation, Inc.
Four Allegheny Center, 5th Floor
Pittsburgh, PA 15212

Other Participating Researchers

Soonmyung Paik, MD; Nan Song, PhD – employed by NSABP Foundation, Inc.

Expected Research Outcomes and Benefits

The expected outcomes of this research are the identification of new therapeutic agents for colon cancer and a greater understanding of the mechanisms responsible for resistance and sensitivity to specific therapeutic agents on the viability of colon cancer cells. Combining information about the response to targeted agents, the results of relevant signaling pathway assays, and the molecular profiles of the cell lines may lead to hypotheses to explain therapeutic resistance. Identification of molecular aberrations in cancer cell lines that correlate with resistance or sensitivity will provide a rationale for selection of patients for specific treatments.

Summary of Research Completed

<u>AIM 1</u>- Identify new agents and agent combinations to optimize colon cancer cell killing.

During this report period, we tested targeted agents with the goal of improving the treatment of colon cancer patients with *KRAS*-mutant tumors. Currently, cetuximab, an anti-EGRFR inhibitor, has been shown to improve overall survival, progression-free survival, and overall response rate

but only for metastatic colorectal patients with *KRAS* wild type (WT) tumors. Unfortunately, mutations in the *KRAS* oncogene are common in colon tumors, occurring in approximately 35%-45% of metastatic colorectal cancer patients. Recent evidence has suggested that combining targeted agents can be an effective strategy for inhibiting the growth or viability of cell lines even when the agents blocked the same pathway.

Cell lines were selected for the testing of targeted agents on the basis of the cells' *KRAS* mutation status and on their subtype as determined by gene expression signatures recently described in the literature. Our in-house data and the published literature have shown that the stem-like subtype of colon cancer has the worst prognosis. Thus, we have selected 5 cell lines (SW480, SW620, HCT 116, C2BBe1, Hs 675.T) with a stem-like gene expression signature for testing of targeted agents. Two other cells lines, which were characterized as inflammatory (NCI-H747, SW837), were selected for comparison to the stem-like cell lines. To determine whether *KRAS* mutation status correlated with response to agents, we included two stem-like cell lines which were *KRAS* WT. The mutation status (0 indicates WT and 1 indicates a mutation) and subtype designations of these cell lines are shown below in Table 1.

Initially, the following inhibitors were evaluated as single agents: an EFGR inhibitor, gefitinib; two MEK inhibitors (selumetinib, MEK162); and two pan ErbB inhibitors (afatanib and neratinib). All these inhibitors have proven to be effective as single agents in other cancers, such as lung and melanoma, and are being tested in clinical trials for other cancers. Cell lines were treated with increasing concentrations of these inhibitors in 3 cellular assays—cell proliferation, cell viability, and clonogenic assays as described below.

Gefitinib showed little to no effect on cell viability in all of the cell lines even at a concentration of 5uM. All cell lines were also relatively resistant to neratinib and afatinib but some cell lines did show reduced viability (Figure 1). The MEK inhibitors, selumetinib and MEK162, had IC50 values below 1uM for the inflammatory cell lines SW837 and NCI-H747, but the stem-like cells were more resistant to these agents with some variation on the level of resistance; however, this resistance was not correlated with KRAS mutations. The cell lines that appeared to be most resistant to the MEK inhibitors were the KRAS WT cell lines C2BBe1 and Hs 675.T (Figure 1).

We further examined whether exposure of colon cancer cells to these inhibitors might induce delayed onset of cell death by using clonogenic assays. Cells were incubated with either medium or inhibitors (0-1.5 µM) for 7 to 10 days followed by staining for colony formation. The inflammatory cell lines (NCI-H747 and SW837) were responsive at high concentrations of neratinib and afatanib as compared to the stem-like cell lines (SW480 and SW620) (Figure 2). With these agents, the clonogenic growth assay results were complementary to those of the cell viability assay. These results suggest that there are differential effects of these inhibitors as single agents in different subtypes of colon cancer cell lines. The MEK and pan ErbB inhibitors were also used to treat the all cell lines, and all cell lines appeared to be sensitive to the combination at the concentrations that were used.

Unpublished data suggested that combining MEK inhibitors with a pan ErbB inhibitor may be more effective in colon cancer cell killing. Thus MEK162 was combined with neratinib or with afatanib and tested in all cell lines in cell viability assays (Figure 3). The combination of a MEK

and pan ErbB inhibitor is effective at decreasing cell viability. For example, at concentrations of 0.5 µM neratinib and 0.062 µM MEK162, the cell viability of the inflammatory cell lines is decreased by about 50%. Interestingly, we observed that this combination is not as effective against the stem-like cell lines (SW480, SW620, HCT 116, C2BBe1 and Hs 675.T). The most resistant cell line was SW480, and cell lines SW620, HCT 116 and Hs 675.T and C2BBe1had intemediate resistance to the combination. The *KRAS* WT cell lines, HCT 116, C2BBe1 and Hs 675.T were also resistant to the combination. Thus resistance is associated with subtype and not with mutation status in these 7 cell lines.

Detailed Methods

For cell viability assays, colon cancer cells (8 x 10^3/well) in 100 µl of growth medium were plated into each well of a 96-well microtiter (Costar: Corning Life Sciences, Cambridge, MA) plate. Cells were allowed to attach for 14 to 18 hours; cells were then exposed to different concentrations of inhibitors for 3 days. The number of viable cells was determined by using the CellTiter-Blue Cell Viability Assay (Promega, WI). The CellTiter-Blue reagent is a buffered solution containing highly purified resazurin, which is an indicator dye to measure the metabolic capacity of cells. Viable cells retain the ability to reduce resazurin into resorufin, which is highly florescent. Nonviable cells rapidly lose metabolic capacity, and do not reduce the indicator dye, and as a result do not generate a fluorescent signal. CellTiter-Blue reagent (20 µl/well) was added to cells, and after a 1 hour incubation the fluorescence ($560(20)_{Ex}$/ $590(10)_{Em}$) was measured using the Tecan Infinite F200 plate reader. Relative cell number was calculated by normalizing the absorbance to that of untreated cells as % of viable cells. Cells were treated with the indicated concentrations of selumetinib, gefitinib, afatinib, neratinib, or MEK162 as shown in Figure 1. DMSO (0.01%) was used as the control treatment. Results are shown as triplicates of mean ± SD.

Cell proliferation was measured by CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, WI). Briefly, colon cancer cells (8 x 10^3/well) in 100 µl of growth medium were plated into each well of a 96-well microtiter plate (Costar: Corning Life Sciences, Cambridge, MA), and after overnight attachment, exposed to inhibitors or vehicle (DMSO) for 72 hours followed by the addition of a novel tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate;PMS. MTS is bioreduced by cells into a formazon product that is soluble in tissue culture medium. The absorbance at 490nm was measured with the Tecan Infinite F200 plate reader according to the manufacturer's recommendations. Relative cell number was calculated by normalizing the absorbance to that of untreated cells.

Clonogenicity assays were conducted in duplicate by plating 4000 cells in a 35 mm plates and after 14 to 18 hours of incubation drugs were added. After 7 days of incubation at 37°C, the plates were gently washed with phosphate buffered saline (PBS) colonies were stained using the Hema 3 Manual Staining System (Fisher Scientific). The plates were scanned, and images were represented in JPG format and placed into figures using the Adobe platform. NCI-H747, SW837, SW480 and SW620 cells were treated with the indicated combination of MEK162 plus gefatinib, afatinib, or neratinib at indicated concentrations for 72 hours. DMSO (0.01%) was used as the control treatment. Results are shown as triplicates of mean ± SD.

Table 1. Mutation Status and Subtype Designations of Selected Cell Lines

Cell Lines	crc.subtypes	BRAF	KRAS	PIK3CA	NRAS
NCI-H747	Inflammatory	0	1	0	
SW837	Inflammatory	0	1	0	
SW480	Stem.like	0	1	0	0
SW620	Stem.like	0	1	0	0
C2BBE1	Stem.like	0	0	0	0
HCT116	Stem.like	0	1	0	0
HS675T	Stem.like	0	0	0	0

Figure 1. Cell viability assay results of 5 agents in 7 selected cell lines

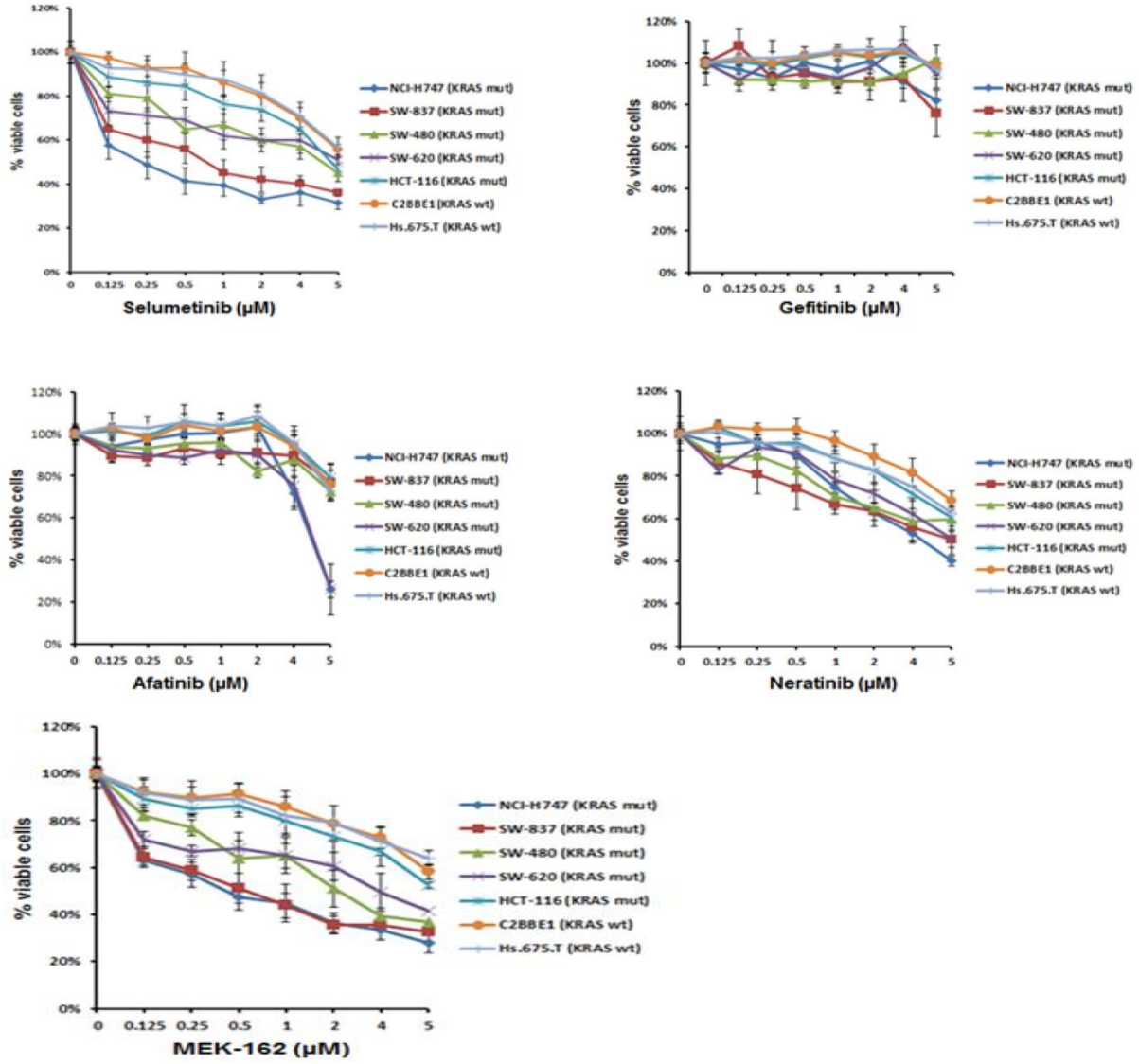

Figure 2. Representative results of clonogenic assay (cell death) in response to various concentrations of neratinib and afatinib

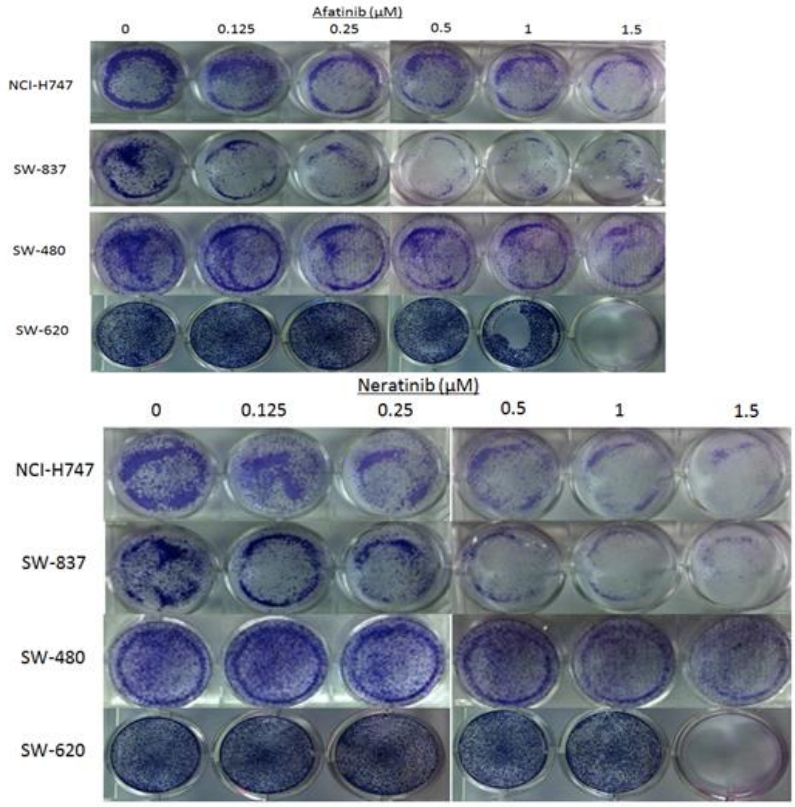
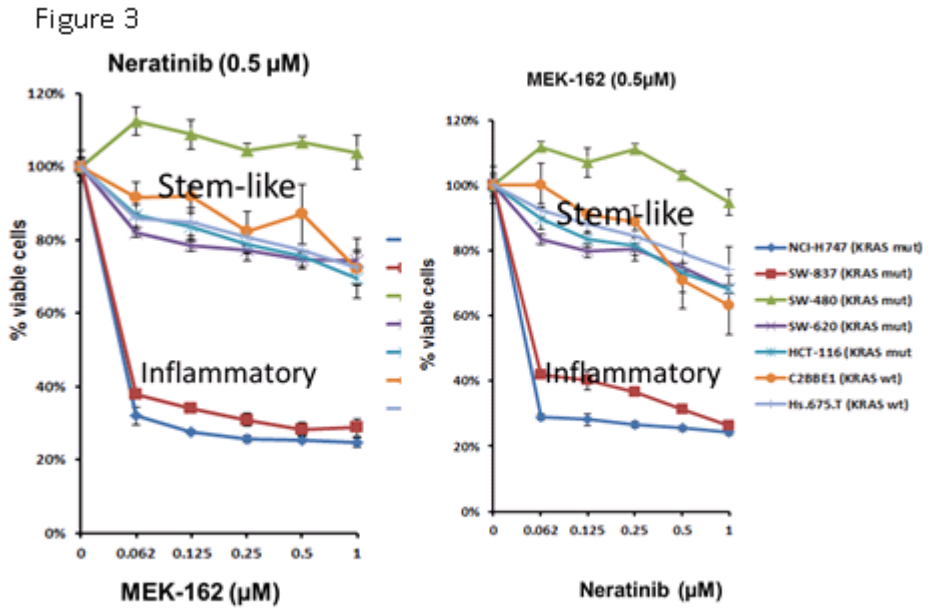
Figure 3. Cell viability following exposure to combinations of neratinib and MEK162.