Annual Progress Report: 2010 Formula Grant

Reporting Period

July 1, 2011 – December 31, 2011

Formula Grant Overview

The Lankenau Institute for Medical Research received $175,518 in formula funds for the grant award period January 1, 2011 through December 31, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Disulfides to Modulate Thiol Homeostasis in Human Colon Cancer Cells – The efficacy of most chemotherapeutic agents and radiation in cancer cells may be limited due to its detoxification by intracellular glutathione (GSH). Our preliminary results have demonstrated that hydroxyethyl disulfide (HEDS) depletes GSH and increases the response of glucose deprived cancer cells to radiation. However, HEDS treatment has only a limited success in increasing the response of rat tumor xenograft to chemotherapy since it is metabolized faster by cells with glucose. The next step in our drug discovery effort is to screen disulfides with different chemical structures that will identify disulfides with better stability than HEDS. These studies will also determine the impact of a low glucose microenvironment, which induces resistance to therapy, on the disulfides mediated depletion of GSH in human colon cancer cells.

Duration of Project

1/1/2011 – 12/31/2011

Project Overview

Recent studies from our group have demonstrated that oxidative pentose cycle deficient or glucose deprived rodent cells and human cancer cells are susceptible to hydroxyethyl disulfide (HEDS) mediated decrease in protein and non protein thiols and sensitization to radiation. Based on these in vitro studies, HEDS was expected to be advantageous in sensitizing solid tumors to radiation since it will specifically sensitize glucose deprived hypoxic tumor cells that are resistant to cancer therapy. However, our preliminary studies (results not shown) have demonstrated that this strategy has only a limited success in increasing the response of rat tumor xenograft to radiation and chemotherapy in vivo since it is metabolized faster by cells with glucose. The next step in our drug discovery effort is to screen drugs with a similar functional (disulfide) group but with different chemical structures to determine the structure activity relationship. This approach will not only identify disulfides with better stability than HEDS but
also will determine the impact of a low glucose microenvironment, which induces resistance to therapy, on the disulfide mediated depletion of glutathione (GSH) in human colon cancer cells.

The planned pilot study will screen twenty disulfides with different chemical structures for their efficacy to alter the redox status of glutathione, and to determine the detoxification of these compounds by human colon cancer cells in the presence and absence of glucose in vitro. The major focus of this project is to determine whether one or more of these disulfide compounds have a better thiol modulation property than hydroxyethyl disulfide.

**Specific Aim 1:** Determine the extent of conversion of disulfides with different chemical structures into sulphydryl compounds (i.e. detoxification) by human colon cancer cells in the presence and absence of glucose.

The conversion of disulfides into sulphydryl will be measured by quantifying free thiols in the extracellular medium produced by bioreduction of disulfides using HPLC/electrochemical detection and/or 5, 5-dithiobis 2-nitrobenzoic acid (DTNB) assays.

**Specific Aim 2:** Determine the effect of these disulfides on intracellular thiol redox in the presence and absence of glucose.

To quantify the effect of these disulfides on intracellular thiols, the cellular extract prepared by sulfosalicylic acid lysis buffer will be used for the analysis of thiols using HPLC/electrochemical detection and/or 5, 5-dithiobis 2-nitrobenzoic acid (DTNB) assays.

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**Expected Research Outcomes and Benefits**

Low oxygen (hypoxia) is prevalent in most solid tumors and plays a major role in the poor outcome of cancer therapy. Hypoxic (low oxygen) cells in solid tumors are also glucose deprived due to disorganized vasculature. Glucose deprived cells are resistant to radiation and certain chemotherapeutic agents. It raises the importance of identifying agents that can target glucose deprived hypoxic cancer cells in solid tumors. Glutathione, an antioxidant, plays a major role in the survival of cancer cells in an oxidative stress environment, which is likely to be caused by low glucose and low oxygen in solid tumors. In contrast, certain disulfides deplete glutathione causing loss of function of proteins and cell death under certain conditions. The FDA has
recently approved a disulfide called glutathione disulfide mimetic (NOV-002) for phase 2/3 clinical trial for cancer in the US. This compound has shown some limited but significant improvement when combined with chemotherapy. NOV-002 is not membrane permeable, which may be the reason for its limited success. We have demonstrated that another disulfide hydroxyethyl disulfide (HEDS) is membrane permeable and specifically targets glucose deprived cancer cells in vitro. These studies suggested that the effectiveness of disulfides in cancer therapy is dependent not only on the disulfide functional group but also on the chemical structure of the compound. Our project will determine the structure activity relationship of several disulfide compounds in targeting glutathione, which plays a major role in the survival of cancer cells during oxidative stress induced by the tumor microenvironment, chemotherapeutic agents and radiation. The planned studies will 1) screen twenty disulfides with various chemical structures in comparison to hydroxyethyl disulfide and NOV-002 for their effectiveness in depleting glutathione and 2) determine the detoxification of these compounds by human colon cancer cells in the presence and absence of glucose. This approach will not only identify disulfides better than HEDS, but will also determine the human colon cancer cells’ ability in the detoxification of these disulfides under glucose deprivation found in solid tumors.

Summary of Research Completed

We tested the effects of these three stable disulfides on glutathione in cancer cells since glutathione oxidation affects the function of several proteins important for the survival and response of cancer cells to therapy. Additionally, we also determined the detoxification capacity of HT29 human colon cancer cells to different concentrations of these three disulfides.

For the completion of the studies proposed in specific Aim 1, we determined the effects of different concentration of three disulfides, which are more stable and yet glucose dependent, in HT29 human colon cancer cells.

As part of Specific Aim 2, we have proposed to determine the effect of disulfides on intracellular glutathione in the presence and absence of glucose. In this project, we tested three disulfides (Bis (4-Methoxyphenyl) disulphide, P-tolyl disulphide and Phenyl disulphide that have exhibited glucose dependent bioreduction by HCT116 and HT29 human colon cancer cells in the presence and absence of glucose. The other disulfides were not studied since these disulfides did not exhibit glucose dependent bioreduction in these cancer cells.

Methods and Designs: We used two established human colon cancer cells (HCT116, HT29) obtained from ATCC, USA. All experiments were carried out at a single concentration of cells (1 million) grown in a 60mm dish with DMEM medium with 10%FCS, 1% penicillin/streptomycin (P/S) and 20 mM HEPES. These cells were rinsed four times with DMEM without glucose (hereafter called as DMEM-G) to remove the residual glucose in the dish. DMEM-G rinsed cells were incubated for four hours with one ml of DMEM-G containing 0 and 25mM glucose. After 4hr glucose starvation, cells were exposed to different concentrations of disulfide for 2hrs at 37°C in a 5% CO₂ incubator to estimate the effect of these disulfides on intracellular non-protein thiol (GSH). Longer incubations were not studied since the conversion was inhibited after 2 hours suggesting that these disulfides may have inhibitory effect on the
bioreductive pathways even in the presence of glucose in these cancer cells. The depletion of thiols in the human colon cancer cells was measured by quantifying the intracellular thiols.

**Results:** We previously demonstrated that hydroxyethyl disulfide was metabolized by human and rodent cells very rapidly in the presence of glucose. In the first six months of the funding period, we identified that 3 disulfides have better stability than the hydroxyethyl disulfide in one of the two cell lines proposed in the project. We therefore determined the stability of these three new compounds (tolyl disulfide and phenyl disulfide) by comparing the metabolic conversion of different concentrations (0, 1, 2, 3, 4, 5mM) of these compounds into monothiols in the other HT29 cells. The results have demonstrated that both Bis (4-Methoxyphenyl) disulphide and tolyl disulfide were converted into a maximum 170μM and 60μM monothiol respectively by HT29 cells after 2 hour incubation in the presence of glucose (Figure 1, 2). On the other hand, phenyl disulfide is converted into 370μM monothiols by HT29 cells after 2 hour incubation in the presence of glucose (Figure 3). The metabolic conversion of these two compounds is lower than the metabolic conversion of hydroxyethyl disulfide (1100 μM, results not shown) under similar conditions. However, all three of these compounds require glucose for metabolic conversion into monothiols (Figures 1-3). The higher stability of these three new compounds compared to hydroxyethyl disulfide suggests that these compounds may be more effective as a chemotherapy agent. Overall, HT29 showed similar trend as that of the HCT116 cells, which was studied in the first six months of the funding period.

Figures 4 and 5 show the effects of different concentrations of bis(4-methoxyphenyl)disulfide on the intracellular glutathione in human colon cancer cells HCT116 and HT29 in the presence and absence of glucose. The results have demonstrated that bis(4-methoxyphenyl)disulfide up to 4mM did not affect the glutathione level in human colon cancer HCT116 cells in the presence of glucose but depleted all the glutathione at 1mM in the absence of glucose (Figure 4). Similar effect was also observed in human colon cancer HT29 cells (Figure 5).

Figures 6 and 7 show the effects of different concentrations of tolyl disulfide on the intracellular glutathione in human colon cancer cells HCT116 and HT29 in the presence and absence of glucose. The results have demonstrated that tolyl disulfide, up to 5mM, did not affect the glutathione level in human colon cancer HCT116 cells in the presence of glucose, but depleted glutathione at greater than 2mM in the absence of glucose (Figure 6). Similar trend was also observed in HT29 human colon cancer cells (Figure 7).

Figures 8 and 9 show the effects of different concentrations of phenyl disulfide on the intracellular glutathione in human colon cancer cells HCT116 and HT29 in the presence and absence of glucose. The results have demonstrated that phenyl disulfide up to 4mM did not affect the glutathione level in human colon cancer HCT116 cells in the presence of glucose but depleted only 60% of glutathione at 1mM in the absence of glucose (Figure 8). Although similar trend was observed in HT29 human colon cancer cells, phenyl disulfide also affected the glutathione level in human colon cancer cells HT29 even in the presence of glucose (Figure 9).

**Significance**

The results have demonstrated that at least three of the fourteen disulfides screened so far have better stability than HEDS and depleted glutathione differently either in glucose rich and glucose...
deprived media. These results suggested that these three new disulfides likely to have better efficacy in killing cancer cells in solid tumors.
Figure 1: Quantification of detoxification of bis(4-methoxyphenyl) disulfide (0, 1, 2, 3, 4, 5mM) after 2 hour incubation with human colon cancer cells HT29 in the presence and absence of glucose. The results have demonstrated that bis(4-methoxyphenyl) disulfide showed a glucose dependent conversion into monothiols at all concentrations tested in vitro.

Figure 2: Quantification of detoxification of tolyl disulfide (0, 1, 2, 3, 4, 5mM) after 2 hour incubation with human colon cancer cells HT29 in the presence and absence of glucose. The results have demonstrated that tolyl disulfide showed a glucose dependent conversion into monothiols at all concentrations tested in vitro.

Figure 3: Quantification of detoxification of phenyl disulfide (0, 1, 2, 3, 4, 5mM) after 2 hour incubation with human colon cancer cells HT29 in the presence and absence of glucose. The results have demonstrated that phenyl disulfide showed a glucose dependent conversion into monothiols at all concentrations tested in vitro.
Figures 4: Effects of different concentrations of bis(4-methoxyphenyl)disulfide on the intracellular glutathione in human colon cancer cells HCT116 in the presence and absence of glucose. The results have demonstrated that bis(4-methoxyphenyl)disulfide up to 4mM did not affect the glutathione level in human colon cancer HCT116 cells in the presence of glucose but depleted all the glutathione at 1mM in the absence of glucose.

Figures 5: Effects of different concentrations of tolyl disulfide on the intracellular glutathione in human colon cancer cells HCT116 in the presence and absence of glucose. The results have demonstrated that tolyl disulfide up to 5mM did not affect the glutathione level in human colon cancer HCT116 cells in the presence of glucose but depleted glutathione at 1mM in the absence of glucose.

Figures 6: Effects of different concentrations of phenyl disulfide on the intracellular glutathione in human colon cancer cells HCT116 in the presence and absence of glucose. The results have demonstrated that phenyl disulfide up to 4mM did not affect the glutathione level in human colon cancer HCT116 cells in the presence of glucose but depleted only 60% of glutathione at 1mM in the absence of glucose.
Figures 7: Effects of different concentrations of bis(4-methoxyphenyl)disulfide on the intracellular glutathione in human colon cancer cells HT29 in the presence and absence of glucose. The results have demonstrated that bis(4-methoxyphenyl)disulfide up to 5mM did not affect the glutathione level in human colon cancer HT29 cells in the presence of glucose but depleted all the glutathione at 1mM in the absence of glucose.

Figures 8: Effects of different concentrations of tolyl disulfide on the intracellular glutathione in human colon cancer cells HT29 in the presence and absence of glucose. The results have demonstrated that tolyl disulfide up to 4mM did not affect the glutathione level in human colon cancer HT29 cells in the presence of glucose but depleted glutathione at greater than 2mM in the absence of glucose.

Figures 9: Effects of different concentrations of phenyl disulfide on the intracellular glutathione in human colon cancer cells HT29 in the presence and absence of glucose. The results have demonstrated that phenyl disulfide depleted glutathione more in the absence of glucose than in the presence of glucose. At greater than 2mM, it also affected the glutathione level in human colon cancer HT29 cells in the presence of glucose.
Research Project 2: Project Title and Purpose

Role of TIMP-4 in Breast Cancer Assessment and Treatment - The planned work will assess the use of a new therapeutic agent to target the triple-negative breast cancers (TNBC) identified as highly aggressive even when diagnosed at a small size. We know from previous work that elevated levels of tissue inhibitor of metalloproteinases-4 (TIMP-4) in TNBC are associated with poor prognosis for disease-free survival. These tumors are highly aggressive and difficult to treat due to lack of targeted therapy and/or resistance to standard therapy. A new agent, which blocks the down-stream effects of TIMP-4, can be the first agent to improve response rates and thereby survival among TNBC patients, a group that contributes disproportionately to the breast cancer associated death rate.

Duration of Project

1/1/2011 – 12/31/2011

Project Overview

The objective for this project is to obtain new information regarding the effects of elevated TIMP-4 levels on tumor behavior. The new information could result in changes in clinical practice to provide a better treatment plan for patients with triple-negative but TIMP-4 positive breast cancer, a group that currently have few effective treatment options available.

In this project we will assess circulating TIMP-4 levels in breast cancer patients by analyzing blood samples collected approximately 3-4 weeks after surgical removal of the tumor (Specific Aim 1). Plasma from the collected blood samples will be analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit. Circulating levels of TIMP-4 will be assessed at several times throughout the initial treatment regimen.

We will also employ a standard animal model of breast cancer to obtain preclinical “proof-of-concept” that blocking TIMP-4 induced signaling can prevent breast tumor growth and progression (Specific Aim 2). The animals will have slow-release pellets containing human TIMP-4 protein implanted into the mammary fat pad (mfp) followed by inoculation of human breast cancer cells. Once palpable tumors have formed, animals will be treated with PI3K-inhibitors alone or in combination with a chemotherapeutic agent. Changes in tumor growth and progression will be followed and compared to vehicle alone. Obtained results will determine if the new PI3K inhibitors could be tested in clinical trials for treatment of TNBCs with elevated TIMP-4.
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Expected Research Outcomes and Benefits
We predict that we will obtain data confirming our initial observation that circulating levels of TIMP-4 are independent of tumor burden and therefore will remain at a similar level post-surgery as pre-surgery (Specific Aim 1). This will attest to the need for TIMP-4 targeted therapy. The results obtained from Specific Aim 2 will provide “proof-of-concept” for our hypothesis that downstream signaling of TIMP-4 can be an effective approach to treat patients with TNBC and elevated TIMP-4 levels. The obtained results will be the foundation of a new R21 application. Further testing to identify an agent that can block the downstream signaling in patients with elevated TIMP-4 levels could help reduce the aggressive behavior and render the tumors more susceptible to conventional chemo-and radiation therapy.

Summary of Research Completed
During the past 6 months additional breast cancer patients were enrolled for specific aim 1, for a total of 13. Of these patients, one has passed due to breast cancer while the others are continuously being followed during their regularly scheduled visits with their oncologist.

These patients have provided us with an EDTA-coated (“purple top”) tube for plasma collection. Samples were drawn prior to 1) initiating therapy, 2) each additional cycle of therapy and 3) follow-up visits. Samples were de-identified by assigning a study number before transfer of samples to the laboratory. Samples were kept on ice and handled at 4°C at all times.

Blood samples were spun at 6,000rpm for 10 minutes at +4°C to separate plasma from blood cells. Plasma was transferred into new tubes, immediately frozen, and stored at -80°C until analysis. At time of analysis, each tube was allowed to thaw on ice and then gently mixed before added into the commercial ELISA coated with a TIMP-4 specific capture antibody (R&D Systems). Samples were added along with known amounts of recombinant human TIMP-4 to generate a standard curve for assessment of TIMP-4 levels in the patient plasma. Following the instructions from the manufacturer, unbound material was washed using the provided wash buffer followed by the addition of a conjugated second TIMP-4 specific antibody and substrate for color development. The absorption at 450nm, obtained using a BIO-TEK Synergy HT plate reader, was used to determine the amount of circulating TIMP-4 per ml of plasma.
Even though early, it seems to be a trend for more effective reduction in circulating levels among patients receiving Adriamycin based therapy over Taxol/Taxotere. Four of the enrolled patients received Taxol (or Taxotere) in combination with Cytoxan (TC) without a sustained reduction to the TIMP-4 levels (Figure 1). An additional four patients received Taxol/Taxotere with other combinations and are too few in number to draw any conclusions from at this time. The most recently enrolled patients have not received more than two cycles at this point and it is therefore too early to assess the effectiveness of her treatment.

The final three patients received Adriamycin in combination with Cytoxan (AC). Two of the patients received this combo from the start of their treatment while the third (LH10-006) started on TC but had a bad reaction and was switched to AC for the remainder of her treatment. The switch resulted in a dramatic reduction in TIMP-4 levels that has remained below the threshold value for normal circulating levels (Figure 2).

During the past 6 months we have completed the testing of our cell-lines and reagents to be used in the animal experiments described for specific aim 2. An external laboratory (RADIL, MO) tested and found the human triple-negative breast cancer cell-line MDA-MB-468 to be free of pathogens. The reagents were tested by PCR for twenty murine viruses and also found to be pathogen-free.

In preparation for the experiments, we had slow-release pellets made (Innovative Research of America, FL). The amount of TIMP-4 used to prepare the pellets was calculated to ensure a daily circulating level of 2,500 pmol/ml. In humans, this level is above the threshold for what is found in non-cancer individuals and 7-8 times higher than what we generally find in non-tumor bearing nude mice.

The problematic part for this portion of the project has been the construction of our new and expanded vivarium. Once the construction was finished, new problems with air handling and humidity were discovered. At the end of last year, more than 6 months delayed, we were finally able to set up our experiments. We have nude mice (nu/nu, Charles River) in our sterile unit and we are currently waiting for results of our treatments.

The animals were delivered and allowed to acclimate to the facility for one week and then ear-tagged for identification purposes so we can distinguish individual tumor growth/progression. Blood samples were collected from randomly chosen animals to establish base-line levels of TIMP-4. All animals had a slow-release pellet implanted in the vicinity of the fourth, left mammary fatpad. Half the colony received a TIMP-4 containing pellet while the other half received a placebo control pallet. Incisions were closed with staples that were removed after one week when the incision had closed. All animals seemed to have tolerated the pellet well and no signs of distress or problems walking have been observed. One week after implanting the pellets, the previously chosen animals were subjected to blood sampling to assure that the TIMP-4 levels were increasing and all animals had $5 \times 10^6$ MDA-MB-468 cells in 100μl injected under the left, fourth nipple near the previously implanted pellet. The cells were 70% confluent when trypsinized and washed twice in phosphate-buffered saline prior to injection.
Reports in the literature had shown that injecting $5 \times 10^6$ MDA-MB-468 cells into the mammary fatpad produces palpable tumors in two weeks. In our hands, most of the animals had tumors at two weeks post-injection. Once the tumors reached 6mm in the largest dimension as determined by calipers, treatment was initiated. We are currently following the tumors for changes in growth.

The anticipated results from this experiment will serve as preliminary data for a long-term grant proposal to extend the studies of TIMP-4 in breast cancer formation, growth and progression.

Figure 1. Effect of Taxol/Taxotere based therapy on circulating TIMP-4 levels in breast cancer patients. At various times, TIMP-4 levels decreased in all four patients only to increase to higher than surgery/pre-chemo values. Solid line indicated cut-off value, determined as mean TIMP-4 level in healthy control + (2 x STDEV), **= time of surgery, * = pre chemo, * = cycle of chemo, * = follow-up appointment
TIMP-4 levels in patients receiving Adriamycin based therapy

Figure 2. Effect of Adriamycin based therapy on circulating TIMP-4 levels in breast cancer patients. All patients obtained and maintained below threshold levels with Adriamycin based therapy, suggesting a possible clinical benefit of Adriamycin therapy for patients with triple-negative and TIMP-4 positive tumors. Solid line indicated cut-off value, determined as mean TIMP-4 level in healthy control + (2 x STDEV), * = pre chemo, * = cycle of chemo, * = follow-up appointment.