Project Title: Influence of the Calorie Restricted Ketogenic diet on the Therapeutic Effects of 3-Bromopyruvate and JHU083 (a prodrug of 6-diazo-5- oxo-L-norleucine, DON) on systemic metastasis in mice.

Sponsor: George W. Yu Foundation for Nutrition and Health, Incorporated
For consideration under Grant Category 2
Principle Investigator: Thomas N. Seyfried, Ph.D.,
Address: Biology Department, Boston College, Chestnut Hill, MA 02467**;** **Thomas.seyfried@bc.edu.**
Participating University: Boston College, 140 Commonwealth Ave., Chestnut Hill, MA 02467
Boston College Tax ID. 042103545.

Research Project Summary

The objective of this research to evaluate the influence of two anti-cancer drugs used alone and in combination with the calorie restricted ketogenic diet (KD-R) on systemic metastasis in the VM-M3met preclinical mouse model. The drugs include; 3-Bromopyruvate (3-BrPA) and JHU083 (a prodrug of 6-diazo-5- oxo-L-norleucine, DON). Both of these drugs have the ability to restrict ATP synthesis. No cancer can survive without energy, as we recently described (1). Consequently, the simultaneous targeting of glucose and glutamine metabolism becomes a viable therapeutic strategy for managing most cancers.

Pedersen and Ko showed that 3-BrPA targets the aerobic fermentation (Warburg Effect) seen in most malignant tumors (2). The Warburg effect is the signature metabolic alteration seen in all cancers and has been linked to the unregulated growth and invasive behavior observed in most cancers (2-6). We have obtained preliminary evidence that 3-BrPA has anti-metastatic potential against our VM-M3met model when injected directly into the tumor. The glutamine analogue, DON, is known to target multiple glutaminases that metabolize glutamine to glutamate (7). We previously found that DON could reduce systemic metastasis in the VM-M3met model, but showed some toxicity when administered under standard dietary conditions (8). Our most recent findings show that a calorie restricted ketogenic diet can improve the therapeutic efficacy of DON while reducing toxicity (9). Recent studies also showed that the DON pro-drug (JHU083) could enhance tumor specific immunity by suppressing the proinflammatory activities of myeloid cells in mouse models of metastatic cancer (10). No prior studies have evaluated the therapeutic efficacy of these two anti-cancer drugs used in combination with the KD-R to manage metastasis in preclinical mouse models.

Metastasis is the general term used to describe the spread of cancer cells from the primary tumor to surrounding tissues and to distant organs and is the primary cause of cancer morbidity and mortality (11-19). It is estimated that metastasis is responsible for about 90% of cancer deaths (20). This estimate has changed little in more than 50 years (21,22). Metastasis involves a series of sequential and interrelated steps. In order to complete the metastatic cascade, cancer cells must detach from the primary tumor, intravasate into the circulatory and lymphatic systems, evade immune attack, extravasate at distant capillary beds, and invade and proliferate in distant organs.(12-15,18,23,24) Metastatic cells also establish a microenvironment that facilitates angiogenesis and proliferation, resulting in macroscopic, malignant secondary tumors. Although systemic metastasis is responsible for the majority of cancer deaths, most research in cancer does not involve metastasis in the *in vivo* state (16,25). That about 1,600 people continue to die each day in the US from cancer, further attests to the failure in managing the disease once it disseminates through the body (26).

A difficulty in characterizing novel drug therapies against metastasis comes in large part from a dearth of animal models that show *systemic* metastasis involving bone marrow and multiple organ systems (16,25,26). The key phenotype of metastasis is that the tumor cells spread naturally from the primary tumor site to secondary locations. Systemic metastasis occurs for the VM-M3met tumor from any implantation site when grown in its natural immunocompetent and syngeneic VM/Dk inbred mouse host (27). Indeed, no other mouse model shows the robust systemic metastatic spread of cancer cells as does the VM-M3met model. The VM-M3met mouse tumor cells express all of the major characteristics seen in human metastatic cancer (11). These cells also express properties of macrophages, which are thought to represent the cellular origin of many human metastatic cancers (11). The VM-M3met cells are engineered to express a firefly luciferase (Fluc)-IRES-EGFP cassette under control of the CMV promoter (27). This allows *in vivo* tumor cell tracking using bioluminescence imaging (28). The VM-M3met model is ideally suited to assess the therapeutic efficacy of drugs and diets that can target systemic metastatic cancers. We recently demonstrated synergistic interactions between the restricted ketogenic diet (KD), hyperbaric oxygen therapy, OAA, and the glutamine inhibitor DON in reducing systemic metastasis in the VM-M3met model (9,29,30).

We recently showed that the KD-R can reduce toxicity and enhance the therapeutic action of anti-cancer drugs (1). It is recognized that the KD-R can reduce blood glucose levels, while elevating blood ketones. This is interesting since many tumors are dependent upon glucose for growth and metastasis, but cannot use ketone bodies (β-hydroxybutyrate) for energy (1,31-33). Our goal is to evaluate the therapeutic efficacy of 3-BrPA and JHU083 when administered alone and in combination with the KD-R on the *in vivo* growth of the VM-M3met tumor cells. No information is available on the therapeutic efficacy of the KD-R administered with 3-BrPA or JH083 against systemic metastasis. The proposed research could provide evidence for a, new and powerful, non-toxic therapeutic strategy for managing systemic metastatic cancer. The proposed research will address issues related to grant category 2 involving <u>biological, chemical, clinical, food science research in nutrition and its effect on disease</u>.

Specific Aims

Aim 1 will measure the influence of 3-BrPA and JHU083 on primary tumor growth, and systemic metastatic spread of the VM-M3met cells. A third group will measure the influence of both drugs used together as a cocktail on primary tumor growth, and systemic metastatic spread of the VM-M3met tumor cells. Organs examined for metastasis will include; liver, lung, kidney, spleen, bone, and brain. In this aim, each drug will be evaluated alone and together for their ability to reduce metastasis.

Aim 2 will determine the influence of the drug cocktail on overall survival of the mice.

Experimental Design, Aim 1

The goal is to determine the influence of 3-BrPA and JHU083 on primary tumor growth, and systemic metastatic spread of the VM-M3met cells under *ad libitum* chow diet and restricted ketogenic diet. The VM-M3met cells will be implanted subcutaneously in the flank of syngeneic inbred VM/Dk mouse host. Termination will be determined by either moribund behavior or flank tumor size (no larger than 2.5 cm^3), as we described previously (31,34). Tumor cell growth and distal invasion will be measured using bioluminescence imaging as we previously described (28). Food intake and body weight are monitored for approximately five days prior to tumor implantation. Bioluminescence imaging and tumor volume measurements will be used to

determine tumor growth once tumors become detectable (usually within five days of implantation). Bioluminescence imaging will also be used to assess distal invasion of the VM-M3met cells according to our published procedures (28,35).

3-BrPA Experiments: 3-BrPA will be injected intratumorally to the mice according to the procedure of Ko and Pedersen (36). Our preliminary results from a previous unpublished study showed therapeutic efficacy only when 3-BrPA was injected directly into the subcutaneous grown VM-M3met tumor (intra-tumor injection). The study will involve two groups of mice: Vehicle injection (phosphate buffered saline, PBS,100 ul) and 3-BrPA injection (10 mg/kg in 100 ul, PBS) under two diet condition, AL chow and KD-R. A total of 80 mice will be used for each group of 40 mice (20 control and 20 treated for each diet x 2 types).

JHU083 Experiments: JHU083 will be obtained from Dr. George Yu. JHU083 will be administered iv (0.1-1.0 mg/kg, every-other-day), as we previously described for our studies with DON (8,9). It will be important to determine if the therapeutic action of JHU083 is similar to what we observed previously with DON. It will be important to evaluate the therapeutic effects of JHU083 at two concentrations 0.1 mg/kg, and 1.0 kg. These concentrations were chosen based on our previous toxicity studies using DON (8,9).). A total of 80 mice will be used for each group of 40 mice (20 control and 20 treated for each diet x 2 types).

Drug Cocktail Experiment: This experiment will determine if therapeutic efficacy against primary tumor growth and systemic metastasis is better using a combination of 3-BrPA and JHU083 than when using each drug alone. It is known that glucose and glutamine are the prime fuels needed for the growth and survival of metastatic cancer cells. 3-BrPA and JHU083 will target glycolysis and glutaminolysis, respectively. The drug concentrations and application used for the cocktail experiment will be determined from the data collected on the therapeutic effects from each drug used alone. A total of 80 mice will be used for each group of 40 mice (20 control and 20 treated for each diet x 2 types).

Aim 2 Experimental Design

Overall survival and quality of life are important for assessing the therapeutic action of cancer treatment. We will measure overall survival in the mice treated with each drug alone and in the mice treated with the three-drug cocktail, as described in Aim 1. Bioluminescence imaging, morbid behavior, and tumor volume measurements will be used to determine overall survival, as we previously described (8,31,34). A total of 80 mice will be used for each group of 40 mice (20 control and 20 treated for each diet x 2 types).

Brief timeline of the progression of research: Research for Aim 1 is expected to require about 18 months for completion. Research for Aims 2 will require 12 months for completion. The entire project should be completed in two years.

Budget

Animals: 320 mice for Experiments. (Experimental 320 mice = 80 cages @ $1.00/day for 60 days = $4,800). Maintenance of 100 VM mice is necessary to generate 200-300 mice for Experiments (Maintenance 25 cages @ $1.00/day for 365 days = $9,125). The total animal cost would include breeding and colony establishment for the proposed studies. This is our best estimate of cost as there is no way to predict how the mice will breed, which could affect whether we can complete all studies within the proposed time frame. It can take a couple of months to breed enough mice needed for the initial studies.

Total: **13,925**

Supplies: $8,000 for tumor implantation, anesthesia, bioluminescence imaging analysis, blood glucose/ketone analysis, cell culture supplies, histology, and equipment maintenance.

Total, **$8,000**

Salaries: Research associate full time salary (12 months) for drug injection, data collection and analysis. Senior Scientist half time (6 months) to oversee all aspects of the project.

Total, **$97,475**.

The total scientific costs are estimated at	**$119,400**
Indirect Costs (10%)	$ 11,940
Total project Costs:	$131,340

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