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The effect of Rhodiola crenulata on a highly metastatic murine mammary carcinoma

Jessica L. Doerner

University of Massachusetts Amherst

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THE EFFECTS OF RHODIOLA CRENULATA ON A HIGHLY METASTATIC MURINE MAMMARY CARCINOMA

A Thesis Presented

by

JESSICA L. DOERNER

MASTER OF SCIENCE

September 2007

Molecular and Cellular Biology
EFFECTS OF RHODIOLA CRENULATA ON A HIGHLY METASTATIC MURINE MAMMARY CARCINOMA

A Thesis Presented

by

JESSICA L. DOERNER

Approved by:

_____________________________
Richard Arenas, Chair

_____________________________
Sallie Smith-Schneider, Member

_____________________________
Kalidas Shetty, Member

Patricia Wadsworth, Interim Director
Program in Molecular and Cellular Biology
Every day you may make progress. Every step may be fruitful. Yet there will stretch out before you an ever-lengthening, ever-ascending, ever-improving path. You know you will never get to the end of the journey. But this, so far from discouraging, only adds to the joy and glory of the climb.

-Winston Churchill
ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to Dr. Richard Arenas and Dr. Sallie Smith-Schneider for giving me such a wonderful opportunity and for teaching me so much along the way. To everyone else with whom I have worked at PVLSI and UMass - I am walking away from this experience a better scientist, but more importantly, a better person. Thank you for all of your support and guidance throughout the last year.
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CHAPTER 1

INTRODUCTION

**Inflammation and cancer**

Prostaglandin endoperoxide synthase, also known as cyclooxygenase (COX), is an important enzyme involved in the conversion of arachidonic acid into prostaglandin H2. The latter compound is a precursor for molecules such as thromboxane (vasoconstrictor), prostacyclin (vasodilator), and prostaglandin E2 (PGE2), which modulates inflammation (Bundred and Barnes 2005). COX-1 and COX-2 are the isoforms of cyclooxygenase and both are expressed in normal and tumor tissues. However, COX-2 is the inducible form of the enzyme and is overexpressed in several types of cancer, including breast cancer (Basu et. al 2004). COX-2 is involved in cellular processes such as angiogenesis, tissue invasion and apoptosis (Williams et al. 2000) and is induced in several ways, including stimulation of the HER-2/neu pathway (Bundred and Barnes 2005). Expression of HER-2 correlates with poor breast cancer prognosis and evidence exists that COX-2 is also associated with a worse prognosis (Gunnarsson et al. 2006). Because COX-2 has such a diverse role it has become a target for therapy.

Inflammation is estimated to contribute to the development of more than 10 percent of all cancers (Marx 2004). The relationship between inflammation and cancer is multi-faceted. There are many factors involved and there is conflicting evidence as far as whether or not inflammation can inhibit cancer, or promote it (Marx 2004). With breast cancer in particular, there is evidence to support the latter. The development and metastatic potential of the disease is aided by the infiltration of macrophages, which release molecules that promote cancer (Marx 2004). One such substance released from
macrophages is reactive oxygen species, which can cause mutations that lead to
carcinogenesis (Marx 2004).

One protein in particular is believed to be responsible for the part that
inflammatory cells play in cancer. NF-κB is a protein that is active in immune cells such
as macrophages. The protein contributes to cancer because it inhibits apoptosis, therefore
not allowing cells with mutations to be removed (Marx 2004). More research on NF-κB
has revealed that it is not necessarily needed for early events. The protein is more
important for the development of malignant cells and is also required for epithelial cells
to undergo the epithelial-mesenchymal transition, which allows them to migrate (Marx
2004). As previously mentioned, COX2 is an enzyme with a well-defined role in
inflammation. NF-κB activates the COX2 gene. This, in turn, helps to sustain
inflammation and promote tumor growth because more immune cells are recruited to the
site (Marx 2004).

A recent study by Sinha et al. (2007) delved deeper into the role of immunity and
inflammation in cancer. Certain cells in the tumor microenvironment can secrete
molecules that promote tumor growth and further inflammation. Interleukin (IL)-1β is a
proinflammatory cytokine that causes myeloid-derived suppressor cells (MDSC) to
accumulate (Sinha et al. 2007). These cells, which are found in cancer patients, cause
immunosuppression by inhibiting T-cell activation (Sinha et al. 2007). A lack of
immunity creates an environment that is very favorable for “malignant transformation
and tumor progression” (Sinha et al. 2007). The authors utilized the 4T1 murine
mammary carcinoma to study the effects of MDSC, inflammation and the progression of
cancer. They first found that MDSC accumulation is induced through several of the prostaglandin E2 (PGE2) receptors, including EP2.

To confirm their *in vitro* findings, the authors carried out *in vivo* studies using both wild-type BALB/c mice and EP2-/- mice. It was found, when compared to wild-type, that mice lacking the EP2 receptor had lower MDSC levels and slower growing primary tumors (Sinha et al. 2007). Another *in vivo* study involved injecting mice with 4T1 cells and concurrently treating with SC58236, a COX-2 inhibitor. The results of this study showed slower primary tumor growth and reduced MDSC accumulation (Sinha et al. 2007).

Overall, these experiments prove that COX-2 and PGE2 have an influence on the progression towards malignancy and also in maintaining an environment that is favorable for the proliferation of malignant cells. The proinflammatory cytokine PGE2 causes an accumulation of MDSC, which, as previously mentioned, inhibits T-cell activation (Sinha et al. 2007). Because immunosuppression results from this inhibition, premalignant cells can make the transition to a malignant phenotype that eventually form a tumor. The host’s immune system never has a chance to intervene because the tumor produces COX2, which sustains MDSC levels, further blocking any immune response (Sinha et al. 2007).

**The 4T1 cell line**

The 4T1 cell line has been utilized for *in vitro* work. The 4T1 cell line is a highly invasive, poorly immunogenic murine mammary carcinoma that arose from a sporadic mammary tumor in a BALB/cfC3H mouse (Hahn et al. 2006). 4T1 cells rapidly proliferate when injected into mice and form highly metastatic tumors, and their
metastatic behavior is thought to imitate human breast cancer. For this reason, the use of 4T1 cells in an animal model is said to correlate with stage IV breast cancer (Xanthopoulou et al. 2005). Primary tumors formed after orthotopic injection of 4T1 cells into the mammary fat pad show areas of necrosis and infiltrating immune cells such as neutrophils (Tufts 4T1 Model).
CHAPTER 2

THE USE OF NATURAL COMPOUNDS IN THE TREATMENT OF BREAST CANCER

Introduction

Phenolics have been extensively studied in the chemoprevention of diseases. These compounds have been shown to reduce the risk of certain diseases, including coronary heart disease, diabetes and cancer (Aboul-Enein et al. 2007). Phenolics have antioxidant properties, which are thought to offer protection from the aforementioned diseases and conditions. When molecular oxygen is metabolized, reactive oxygen species (ROS) form. They are found in multiple forms including hydrogen peroxide, singlet oxygen and superoxide anion radical (Waris and Ahsan 2006). A balance between ROS and antioxidants normally exists in aerobic cells. Either because of more production of ROS, loss of antioxidants, or a combination of both, ROS become cytotoxic to cells (Waris and Ahsan 2006).

Reactive oxygen species can have multiple effects on the cell, many of which are thought to lead to cancer. DNA can be damaged by ROS. This, in turn, leads to mutations as a result of the division of cells with unrepaired damage. If DNA damage occurs in oncogenes and/or tumor suppressors, carcinogenesis can result (Waris and Ahsan 2006). The types of mutations caused by ROS are numerous and it has been established that ROS can modify all four bases (Waris and Ahsan 2006). There are certain modifications that are more common than others and some that are more apt to lead to mutations. However, because of the array of modifications that can be produced
by ROS, it is challenging to determine the incidence and specificity of the resulting mutations (Waris and Ahsan 2006). Studies such as the one done by Aboul-Enein et al. (2007) have found phenolic compounds to be effective antioxidants because of their ability to scavenge free radicals.

There have been many studies done investigating phenolic-containing natural products and their effects on cancer. One such study examined the possible benefits of Mediterranean diets. It is thought that olive oil may protect against certain cancers, including breast cancer. However, the exact mechanism by which it exerts these potential anti-cancer effects is largely unknown (Menendez et al. 2007). The effects of phenolic compounds from extra virgin olive oil were used on several cell lines, including HER2 expressing mammary carcinoma MCF7s, to further elucidate the possible protective effects of olive oil.

Approximately 56 to 84 percent of the fatty acids that make up olive oil are $\omega-9$ MUFA (monounsaturated fatty acid) oleic acid. This composition, along with the many phenolic compounds present, may aid in the protective effects of extra virgin olive oil (Menendez et al. 2007). As previously mentioned, there is a poorer prognosis for breast cancer patients who overexpress HER2. Also associated with this type of cancer are shorter relapses and lower survival rates; therefore preventing and treating these cancers is even more challenging (Menendez et al. 2007). The study carried out by Menendez et al. (2007) focused on one phenolic compound in particular because it was found to inhibit cell growth in a dose-dependent fashion. Oleuropein aglycone had the aforementioned effects on every cell line tested, even those that were stably transduced to overexpress HER2 (Menendez et al. 2007). Along with its effects on cell viability, oleuropein
aglycone had interesting effects on cell death. More death was seen in MCF-7 cells expressing HER2 (“forced expression”) after treatment with oleuropein aglycone as compared to HER2-negative MCF-7 cells after treatment with the same phenolic compound (Menendez et al. 2007). These results led the authors to conclude that there is a relationship between HER2 overexpression and susceptibility to effects of oleuropein aglycone (Menendez et al. 2007).

Trastuzumab (Herceptin) is a monoclonal antibody that is given to patients who have HER2 overexpressing breast cancers. It is very common for patients treated with this drug to eventually become resistant to it (Menendez et al. 2007). For this reason, it is important to investigate ways to prevent or reduce this resistance. Menendez et al. (2007) investigated the combination of oleuropein aglycone and trastuzumab. It was found that trastuzumab was more effective at inhibiting growth when given with the phenolic compound, suggesting a synergistic effect (Menendez et al. 2007). Another significant finding from this study was that oleuropein aglycone had an effect on the concentration of HER2 extracellular domain (ECD) found in the media of cultured cells. HER2 is thought to be activated when its ECD is cleaved; trastuzumab works to prevent this cleavage event (Menendez et al. 2007). When cells were treated with the phenolic compound, the concentration of HER2 ECD was lower as compared to the control (Menendez et al. 2007). Yet another finding of this study was that the combination of oleuropein aglycone and trastuzumab was able to reduce the amount of HER2 receptors on the surface of breast cancer cells (Menendez et al. 2007).

A Mediterranean-style diet that is olive-oil based has been shown through epidemiological studies to be linked to lower incidences of certain chronic diseases and
breast cancer (Menendez et al. 2007). It is likely that the phenolic compounds found in extra virgin olive oil contribute to its protective effects (Menendez et al. 2007). Phenolics are known to be one of the active ingredients in Rhodiola. The 4T1 cell line utilized in the current work does express HER2 and it appears as though Rhodiola is able to affect death and protein expression.

The majority of research published on Rhodiola has been on the species *Rhodiola rosea*. Majewska et al. (2006) investigated the effects of Rhodiola on the leukemia cell line, HL-60. The goal of the study was to “define the anticancer effect of the extract” (Majewska et al. 2006). The investigators treated cells with different concentrations of Rhodiola and also carried out several time points. Cell survival, represented by propidium iodide staining, was decreased after Rhodiola treatment. The study using *Rhodiola rosea* utilized cell cycle analysis by flow cytometry and found that the percentage of cells in S phase was increased after treatment whereas G1 and G2/M phase cells were decreased (Majewska et al. 2006). The authors also looked at the mitotic index using a DAPI staining technique. Depending on the concentration used, cell division was reduced or inhibited completely by *Rhodiola rosea*.

Another natural compound that has been investigated in regards to cancer is lactic acid. It is believed that the incidence of colon cancer is lowered by consumption of large amounts of fermented milk and yogurt (Rafter 2002). Most of the studies done using lactic acid bacteria focus on colorectal cancer and the exact mechanism(s) by which lactic acid bacteria exert their effects are unknown (Rafter 2002). Ideas as to how they function include the ability to alter metabolic activities of microflora in the intestine as well as the
production of antitumorigenic compounds and the degradation of carcinogens (Rafter 2002).

**Materials and Methods**

**Cell Culture**

4T1 cells were cultured in RPMI-1640 medium (ATCC bioproducts) containing 1 mM sodium pyruvate, 2 mM L-glutamine, 4500 mg/L glucose, 10 mM HEPES, and 1500 mg/L sodium bicarbonate. The media was supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin. Cells were incubated at 37° C with 5% CO₂.

**FACS Calibur Flow Cytometry**

In order to analyze effects of Rhodiola treatment on 4T1 cells, death assays using flow cytometry were carried out. A total of 50,000 cells were seeded in six well plates and incubated for two days, at which point they were approximately 50% confluent. Before treatment, cells were washed with 1x PBS and then 3 mL fresh media was added to each well. Rhodiola treatments and vehicle (10% EtOH) were spiked in to the media (Table 2.1). Treatments were carried out for 24 hours. At the 24 hour time point, the media from each well was transferred to a labeled conical tube in order to retain the dead cells. The cells were then washed with 1 mL of 1x PBS, which was subsequently transferred to the conical tube containing the media. A volume of 0.5 mL trypsin was then added to each well. After cells were trypsinized, the media/PBS mixture was used to take up the cells from each well. The cells were then centrifuged for 5 minutes at ~1000 xg. The supernatant was aspirated and the cell pellets were resuspended in 1 mL
of 1x PBS and transferred to plastic tubes. 1 µL propidium iodide was added to each sample, except for the blank control (used for calibration). Samples were left in the dark for 10 minutes and then analyzed using the FACS Calibur flow cytometer. The assay was done in triplicate and repeated three times.

Death assays were also done using a combination of Rhodiola and lactic acid. The method was the same as above except that 1% solutions of both sodium lactate (control) and lactic acid (treatment) were added in addition to the Rhodiola and ethanol (vehicle control) in either a 1:50 or 1:25 ratio (Table 2.2). Again, each trial was done in triplicate and repeated three times.

Tritiated Thymidine Incorporation Assay

In order to examine 4T1 proliferation after Rhodiola treatment, a tritiated thymidine assay was done. A total of 10,000 cells were plated in each well of a six well plate. Cells were allowed to attach to the culture dish overnight. The cells were next washed with 1X PBS and then 2 mL of fresh media was added to each well. Vehicle and treatments were added to the media along with 1 µL/mL tritium (Perkin Elmer). Treatments were carried out for 24 hours, after which the media was aspirated and cells were washed with 1 mL 1X PBS. The PBS was removed and 1 mL 10% trichloroacetic acid (TCA) was added to each well. The TCA was removed and cells were washed again with 1 mL 1X PBS. Finally, 1 mL 0.1% SDS/0.1 N NaOH was added to the cells and allowed to sit at room temperature for five minutes. A volume of 5 mL scintillation fluid and 200 µL glacial acetic acid was added to scintillation vials. A volume of 800 µL of the cell lysate was added to the scintillation vial which was subsequently capped and
labeled. Vials were placed in the liquid scintillation analyzer (Perkin Elmer Tri-Carb 2900 TR) and data was collected using QuantaSmart software (Flag 11).

Statistical Analysis

Results were analyzed using a one factor analysis of variance (ANOVA) and Post-hoc tests, where appropriate, were performed by Bonferroni’s t test, where the mean squared error term in the ANOVA table was used as the point estimate of the pooled variance (Graphpad Software, Inc. San Diego, CA).

Results

There was a clear dose-dependent response to Rhodiola treatment. Posthoc analysis using Bonferroni's t-test revealed that three of the five treatment groups (1:75, 1:50 and 1:25) cause a significant amount of death as compared to the vehicle (control) treatment (Figure 2.1). Importantly, the concentrations of Rhodiola used in this assay are physiologically relevant and therefore would be applicable in an in vivo study. The combination studies including Rhodiola and lactic acid revealed that there does not appear to be an additive or synergistic effect with combined therapy in either a 1:25 or 1:50 ratio (Figures 2.2 and 2.3 respectively).

Rhodiola crenulata did not appear to have a significant affect on proliferation. There was a great amount of variability in the cpm values obtained and there was no dose-dependent response (Figure 2.4). It is very possible that Rhodiola crenulata significantly effects death yet has no role in cell proliferation. Another reason why there may not have been interesting or significant finding is because it was unknown that the
tritium being used in the laboratory was supposed to be used within one month of receiving it. After this amount of time the tritium is degraded because of a cleavage event that produces radicals. The aliquot of tritium I used for my assays had been stored for much longer than the recommended month and so it is highly likely that my results were skewed for this reason.

**Discussion**

I have shown that Rhodiola is successful in causing death in the highly invasive and proliferative 4T1 cell line. It is plausible to believe that the phenolic compounds present in *Rhodiola crenulata* are responsible for the effects seen above.

In past work, the extract has been fractionated and assays have been carried out using the different components. However, assays using 4T1 cells with *Rhodiola crenulata* were done using only the whole extract. These results are promising because they support the idea that a whole plant extract is effective, as compared to only using fractions of the extract. Studies have found that using the whole cell extract can have more of a cytotoxic effect than using just active compounds alone (Majewska et al. 2006). With fractionation, it is very possible to lose the synergistic effect that may be present when the compounds are together in the whole cell extract (Majewska et al. 2006).

As previously mentioned, Majewska et al. (2006) investigated the effects of *Rhodiola rosea* on HL-60 leukemia cells. Though the authors did not look at proliferation using a tritiated thymidine assay, they were able to examine the effects on
proliferation by observing the mitotic index. The authors found that cell division was totally inhibited after the addition of the two highest concentrations of the plant extract. This data shows that Rhodiola, albeit a different species, is able to influence proliferation. This leads me to believe that *Rhodiola crenulata*, in some capacity, may affect proliferation. In order to accurately assess the effects of *Rhodiola crenulata* on 4T1 proliferation, the assay needs to be repeated using a new radiochemical, to ensure that the thymidine molecule is not degraded.

In conclusion, the death assay by flow cytometry shows that upon treatment with Rhodiola, death is induced in 4T1 cells. The assay explained above using a propidium iodide stain only shows that cells are dying, however, it does not reveal whether it is by apoptosis or necrosis. Further analysis by flow cytometry using different stains such as Annexin V-FITC, would reveal such information. Further studies need to be done to investigate the combination therapy of Rhodiola and lactic acid. More concentrated solutions may reveal different results.
Table 2.1 Volumes and concentrations of *Rhodiola crenulata* used for *in vitro* assays.

Amount of *Rhodiola* added (µL)

<table>
<thead>
<tr>
<th>Amt. of media</th>
<th>500 µL</th>
<th>3 mL</th>
<th>4 mL</th>
<th>8 mL</th>
<th>Conc. (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control 10% EtOH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>120</td>
<td>160</td>
<td>320</td>
<td>0.067</td>
<td></td>
</tr>
<tr>
<td><strong>1:150 Rhodiola</strong></td>
<td>3.33</td>
<td>20</td>
<td>26.7</td>
<td>53.3</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>1:100 Rhodiola</strong></td>
<td>5</td>
<td>30</td>
<td>40</td>
<td>80</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>1:75 Rhodiola</strong></td>
<td>6.7</td>
<td>40</td>
<td>53.3</td>
<td>106.7</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>1:50 Rhodiola</strong></td>
<td>10</td>
<td>60</td>
<td>80</td>
<td>160</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>1:25 Rhodiola</strong></td>
<td>20</td>
<td>120</td>
<td>160</td>
<td>320</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Table 2.2 Volumes and concentrations of Rhodiola and lactic acid (separate and in combination) used for *in vitro* assays.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amount added</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:25 10% Ethanol (vehicle)</td>
<td>120 µl</td>
<td>0.4</td>
</tr>
<tr>
<td>1:25 Rhodiola</td>
<td>120 µl</td>
<td>0.4</td>
</tr>
<tr>
<td>1:25 Sodium lactate</td>
<td>120 µl</td>
<td>0.4</td>
</tr>
<tr>
<td>1:25 Lactic acid</td>
<td>120 µl</td>
<td>0.4</td>
</tr>
<tr>
<td>1:25 Ethanol + Sodium lactate</td>
<td>120 µl</td>
<td>0.4</td>
</tr>
<tr>
<td>1:25 Rhodiola + Lactic acid</td>
<td>120 µl</td>
<td>0.4</td>
</tr>
<tr>
<td>1:50 10% Ethanol (vehicle)</td>
<td>60 µl</td>
<td>0.2</td>
</tr>
<tr>
<td>1:50 Rhodiola</td>
<td>60 µl</td>
<td>0.2</td>
</tr>
<tr>
<td>1:50 Sodium lactate</td>
<td>60 µl</td>
<td>0.2</td>
</tr>
<tr>
<td>1:50 Lactic acid</td>
<td>60 µl</td>
<td>0.2</td>
</tr>
<tr>
<td>1:50 Ethanol + Sodium lactate</td>
<td>60 µl</td>
<td>0.2</td>
</tr>
<tr>
<td>1:50 Rhodiola + Lactic acid</td>
<td>60 µl</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Figure 2.1 Response of 4T1 cells to varying concentrations of *Rhodiola crenulata*. Cells were treated for 24 hours with the ratios of Rhodiola or vehicle described in table 2.1, harvested, stained with propidium iodide and analyzed by FACScalibur flow cytometry.

* p < 0.05, significantly different from control group using Bonferoni’s *t*-test following one-way ANOVA.
Figure 2.2 Response of 4T1 cells to a 1:25 ratio of Rhodiola, lactic acid and combination. Cells were treated for 24 hours with the ratios of Rhodiola, lactic acid, or vehicle described in table 2.2, harvested, stained with propidium iodide and analyzed by FACScalibur flow cytometry. Vehicle bars (left to right – 10% EtOH, 1% sodium lactate, combination), treatment bars (left to right – Rhodiola, 1% lactic acid, combination).
Figure 2.3 Response of 4T1 cells to a 1:50 ratio of Rhodiola, lactic acid and combination. Cells were treated for 24 hours with the ratios of Rhodiola, lactic acid, or vehicle described in table 2.2, harvested, stained with propidium iodide and analyzed by FACScalibur flow cytometry. Vehicle bars (left to right – 10% EtOH, 1% sodium lactate, combination), treatment bars (left to right – Rhodiola, 1% lactic acid, combination).
Figure 2.4 Effect of *Rhodiola crenulata* on 4T1 cell proliferation. 10,000 cells were plated, treated and 1 µL/mL tritium was added to each well. Treatments were carried out for 24 hours and cpm data was collected using a liquid scintillation counter.
CHAPTER 3
THE ROLE OF CYCLOOXYGENASE-2 IN BREAST CANCER

Introduction

Numerous studies have investigated the effects of COX-2 inhibitors in breast cancer. Celecoxib (brand name Celebrex) is a non-steroidal anti-inflammatory drug (NSAID) originally used to treat rheumatoid arthritis and osteoarthritis (National Institutes of Health Medline). Prostaglandins, which are produced by cyclooxygenase, promote inflammation, fever and pain. The function of NSAIDs is to block COX enzymes, therefore reducing the production of prostaglandins and consequential fever, pain and inflammation (MedicineNet). It has been found that celecoxib may have a role in the treatment of breast cancer.

Basu et al. (2004) found that treatment with celecoxib reduced mammary gland tumor burden in MMTV mice. The investigators attribute tumor reduction to apoptosis of tumor cells in vivo (Basu et al. 2004). Tumor lysates from control and celecoxib treated mice were run on SDS gels. The blots showed that celecoxib reduced the amount of phosphorylated Akt, which promotes cell survival through several pathways. The proapoptotic protein Bax was upregulated after treatment, whereas the antiapoptotic protein Bcl-2 had decreased expression after celecoxib treatment (Basu et al. 2004). This study also addressed the role of COX-2 in angiogenesis. The authors found that treatment lowered levels of VEGF in mammary gland tumors and a decreased amount of blood vessels were seen in tumor sections from celecoxib treated mice (Basu et al. 2004).
Another study by Basu et al. in 2006 found that the highly invasive breast cancer cell line MDA-MB-231, which overexpresses COX-2, was able to form vascular channels. Treatment with celecoxib was able to inhibit channel formation.

Studies have also shown that combination treatments that include celecoxib have been efficient in treating breast cancer. Liu et al. (2003) studied the effects of celecoxib and radiation. As compared to celecoxib treatment alone, combination therapy resulted in delayed tumor growth (Liu et al. 2003). Bundred and Barnes (2005) discuss the potential of the combination of aromatase inhibitors and COX-2 inhibitors. As mentioned previously, cyclooxygenase converts arachidonic acid into prostaglandin H2. The latter compound is a precursor for several molecules, including prostaglandin E2 (PGE2). Increased cox-2 mRNA expression therefore results in high levels of PGE2. In turn, PGE2 leads to increased cyp19 expression, which amplifies aromatase activity. Because of the relationship between cyclooxygenase and aromatase, both inhibitors have aromatase as a common target (Bundred and Barnes 2005). This could be beneficial in the treatment and/or prevention of breast cancer. Evans et al. (2004) investigated the combination of celecoxib and amiloride, a plasminogen activator inhibitor. The investigators found that this combination showed a synergistic effect on the inhibition of cellular invasion in vitro.

As shown, there has been a significant amount of work done with celecoxib in relation to breast cancer whereas work with Rhodiola is on the rise. It is thought that Rhodiola exhibits its effects through multiple pathways (Smith-Schneider, personal communication.). What is of interest to me is the belief that this species may have an effect on COX, which is overexpressed in many cancers (Smith-Schneider,
personal communication). Data shows that treatment with Rhodiola can inhibit COX activity almost completely in MCF-7 and MDA-MB-231 cells (as measured through PGE$_2$ levels). The immortalized cell lines, 76N tert and 76N p53 239, also showed decreased COX activity after Rhodiola treatment (Smith-Schneider, personal communication). There is much more data from work done with *Rhodiola crenulata* that explores other possible pathway targets and cellular functions, however, the effect on COX-2 is the focus of this chapter.

**Materials and Methods**

Use of Celecoxib (Celebrex) for *in vitro* studies

My original intent was to investigate the effects of celecoxib in combination with Rhodiola. Using the same death assay protocol listed above, I did FACS analysis using combinations of Rhodiola and celecoxib. Unfortunately, there were several problems with the celecoxib, which made *in vitro* assays very difficult. To start with, I was working with the powder obtained from celecoxib capsules. Without the use of sophisticated techniques to separate the active compound from the bulk powder, I was only able to treat cells using the mixture contained within the capsule. Secondly, upon visualization of my plates I found that the drug was falling out of solution in media. This phenomenon was also seen during FACS analysis. This presented a considerable problem, as it was difficult to assess whether the drug was having any effect at all. For these reasons, combination treatments with celecoxib would not be feasible and were not continued.
Western Blot Analysis

The next step was to examine the effects of Rhodiola on COX-2 expression through Western blot analysis. Cells were seeded in 100 mm culture dishes and allowed to grow to approximately 50% confluency, at which point they were washed with 1x PBS. A volume of 8 mL fresh media was added to each culture dish and vehicle and Rhodiola treatments were added (Table 2.1). Treatments were carried out for 24 hours. Cell lysates were prepared by washing the cells with 1x PBS and adding 400 µL RIPA buffer (with 1:100 phophatase inhibitors and 1:500 protease inhibitors). Plates were put on ice for five minutes after which cells were removed using a cell scraper. Lysates were collected, placed in eppendorf tubes and subsequently put on ice for ten minutes. Lastly, the lysates were centrifuged at 15,000 xg at 4°C for 15 minutes. The supernatant was transferred to a new tube and kept at -20 °C. Protein concentrations were measured using a BCA assay. Protein standards were made using bovine serum albumin and RIPA buffer as a diluent. Manufacturer’s instructions were followed for the remainder of the protocol (Pierce BCA Protein Assay Kit). Samples were prepared by adding loading buffer to 50 µg of protein and boiling for five minutes.

The appropriate percentage gels were cast using 30% acrylamide (Sigma) and samples were loaded along with a protein standard. The gel was run at 100 volts and then transferred at 100 volts to either PVDF or nitrocellulose membrane. The membranes were blocked in the appropriate blocking reagent (10% milk in TBST or 3% BSA in TBST) for one hour at room temperature. Following the blocking, the membranes were washed quickly and placed in primary antibody (Cayman Chemical COX-2 1:200) overnight at 4 °C. Following primary antibody incubation blots were washed 3x 15
minutes with TBST (0.1% tween). Secondary antibody (Pierce goat anti-rabbit 1:5000) was added to 10 mL TBST and placed on the membrane(s) for one hour and rocked at room temperature. Blots were then washed 3x 15 minutes with TBST after which luminol (SantaCruz luminol reagent, Pierce SuperSignal West Pico) was added for 1-5 minutes and blots were exposed to film (Kodak Biomax/MR) (Figure 3.2). Following exposure, blots were washed 3x 15 minutes in TBST and using the aforementioned method, probed for a loading control (actin, vinculin or tubulin). Westerns for COX-2 expression were run three separate times.

Quantitative Real Time Polymerase Chain Reaction

4T1 cells were plated in six well plates and allowed to attach to the culture dish overnight. Cells were then washed with 1x PBS, fresh media was added, and vehicle and treatments were spiked in to the media. Following 24 hours of treatment, RNA was isolated using an acid-phenol extraction procedure according to the manufacturer’s instructions (Trizol, Invitrogen, Carlsbad, CA). The RNA pellet was re-suspended in nuclease free water and quantified by UV spectrophotometry. The SYBR® Green QRT-PCR Master Mix 1-step kit was used for qRT-PCR. RNA was diluted to a final concentration of 100 ng/µL and primers (for COX-2 and GAPDH) were diluted to 5 nM. The total reaction volume was 10 µL, which included 1 µL RNA. Each treatment was done in triplicate.
Statistical Analysis

Results were analyzed using a one factor analysis of variance (ANOVA) and Post-hoc tests, where appropriate, were performed by Bonferroni’s t test, where the mean squared error term in the ANOVA table was used as the point estimate of the pooled variance (Graphpad Software, Inc. San Diego, CA). For Western blots, densitometry was done using ImageJ software (NIH).

Results

Studies using Celecoxib were not successful because, as previously mentioned, the drug fell out of solution and therefore it was difficult to assess whether or not it was active. A very distinct population was visible upon graphing the results of the flow cytometry analysis (Figure 3.1). Combined with visualizing the plates and observing small specks throughout, it was determined that this “debris” population represented the Celecoxib particles. Through Western blot analysis, I found that Rhodiola treatment did not have any significant effect on COX-2 expression (Figure 3.3). The qRT-PCR showed similar results to the Western. Rhodiola treatments did not have a significant effect on mRNA transcript levels (Figure 3.4).

Discussion

Previous data from the Smith-Schneider lab using different cell lines has shown that COX-2 protein expression is induced upon Rhodiola treatment. However, when the
activity of COX-2 was investigated, the opposite effect was seen; Rhodiola was able to inhibit COX activity (Smith-Schneider, personal communication). It is possible that the effect observed on COX-2 expression is cell-type dependent and Rhodiola simply does not influence COX-2 expression in 4T1 cells. It would have been beneficial for Rhodiola to knock-down COX-2 protein expression. However, it is promising that it does not appear to be inducing it since there is evidence that overexpression of COX-2 correlates with a worse prognosis. The qRT-PCR data is consistent with that of the Western blot. Although the graph appears to show somewhat of a dose-dependent increase in mRNA transcript levels as Rhodiola concentrations increase, there is no significance amongst any of the groups. The qRT-PCR should be repeated several more times to increase the n since the data presented is only representative of one run with each treatment done in triplicate.
Figure 3.1 Effect of Rhodiola and celecoxib combination treatments on 4T1 cells. The circled area represents the population that arose after FACScalibur analysis which is assumed to be the drug falling out of solution.
Figure 3.2 Representative Western blot probed for cyclooxygenase-2 (upper bands) and actin (lower bands).
Figure 3.3 Effect of Rhodiola treatments on COX-2 expression. Cells were treated for 24 hours with varying concentrations of Rhodiola and then lysed in RIPA buffer. Lysates were run on polyacrylamide gels, transferred to PVDF or nitrocellulose membranes and probed for COX-2.
Figure 3.4 Effect of Rhodiola treatment on COX-2 mRNA levels. 4T1 cells were plated, treated for 24 hours and RNA was isolated using Trizol. All treatments were done in triplicate and normalized to GAPDH.
CHAPTER 4
THE ROLE OF *RHODIOLA CRENULATA* IN MIGRATION, INVASION AND METASTASIS

**Introduction**

There has not been a considerable amount of research done investigating the role of *Rhodiola crenulata* in invasion and metastasis. For this reason, the 4T1 cell line is particularly useful because it proliferates so rapidly and is known *in vivo* to metastasize within weeks of the formation of primary tumors. My goal was to carry out an *in vitro* study to characterize the effects of this species of Rhodiola on cellular processes such as proliferation and death. My other major objective was to uncover the possible mechanism(s) through which *Rhodiola crenulata* acts to influence invasion and metastasis.

In an effort to identify proteins involved in 4T1 migration and invasion, several key metastatic proteins were focused on. The matrix metalloproteinase (MMP) family of proteins has been found to play a role in cancer invasion and metastasis (La Rocca et al. 2004). It is well known that degradation of the extracellular matrix is necessary for the invasive growth of cancer (La Rocca et al. 2004). There are four groups of MMPs; one such group is the gelatinases. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are able to break down a key component of the extracellular matrix, type IV collagen (La Rocca et al. 2004). It is now believed that these gelatinases may have specific roles in breast cancer tumorigenesis because of interactions with tumor suppressor genes and oncogenes (La Rocca et al. 2004). These proteins have clinical significance because they have
recently been linked to disease prognosis. In patients with node negative breast cancer, the prognosis is believed to be better if the patient is negative for MMP-2. Conversely, there is a poorer overall survival rate correlated with higher plasma levels of MMP-9 (La Rocca et al. 2004).

La Rocca et al. (2004) found that when compared to control samples, serum samples from breast cancer patients showed higher activity of both MMP-2 and MMP-9. Importantly, the proenzymatic forms of both MMP-2 and MMP-9 had a stronger intensity in sera from breast cancer patients as compared to control sera (La Rocca et al. 2004). Also, most samples from patients with carcinoma showed two lytic bands (MMP-9 dimers) that were previously seen in a colon cancer study; these bands were mostly absent from control samples (La Rocca et al. 2004). Interestingly, an association between MMP-2 and MMP-9 activity and tumor size, stage and node involvement was not found. A positive correlation was seen between c-erbB-2 overexpression and gelatinolytic levels, whilst a negative correlation with ER expression was observed (La Rocca et al. 2004). It is believed overexpression of c-erbB-2 in certain breast cancers may lead to higher production of gelatinases (La Rocca et al. 2004).

Because there is strong evidence implicating matrix metalloproteinases in cancer invasion and metastasis, this family of proteins was focused on in the following investigation.
Materials and Methods

Western blot analysis

Following the same protocol described above, Western blot analysis was done in order to examine protein levels of several MMP family members after treatment with *Rhodiola crenulata*. Polyacrylamide gels were run and protein was transferred to either PVDF or nitrocellulose membrane. Blots were probed overnight with primary antibodies (MMP-2, MMP-3 [rabbit monoclonal, Epitomics], MMP-9 [rabbit polyclonal, Cell Signaling]). After washing, secondary antibodies were added for one hour after which blots were washed and subsequently exposed to film.

Scratch wound assay

A very simple way of assessing the effects of treatment on migration is the scratch wound assay. Approximately 250,000 cells were seeded in six well plates and grown to 100% confluency. At that time, a 10 μL pipette tip was used to create a “scratch” down the middle of each well. In order to remove any debris created by this process, the cells were washed with 1x PBS and then 3 mL fresh media was added to each well. Vehicle and Rhodiola treatments were added (Table 2.1) and plates were incubated for 24 hours. Representative images were taken before and after creating the scratch. After 24 hours of treatment, images of each well were captured.
**Matrigel invasion assay**

A more complex method exists for studying invasion. Growth Factor Reduced Matrigel Invasion Chambers were purchased from BD Biosciences. Control inserts without Matrigel were also purchased. When the assay was ready to be done, the invasion chambers first had to be re-hydrated. A volume of 500 µL serum free media was added to each invasion chamber and the plate was then incubated at 37° C for two hours. Media was aspirated from a confluent plate of 4T1 cells and then washed with 1x PBS. Cells were trypsinized and taken up in regular media (containing serum) in order to stop the action of the trypsin. Cells were centrifuged at 1,000 xg for five minutes. The pellet was then re-suspended in PBS to remove any media containing serum and centrifuged again at 1,000 xg for five minutes. The pellet was re-suspended in approximately 5 mL serum free media and cells were counted using a hemacytometer.

One portion of the protocol that had to be optimized was the number of cells plated. The manufacturer’s instructions suggest seeding 25,000 cells in each invasion chamber. I found after several attempts that this number of cells was too small. The percentage of cells that migrates through the control inserts (no matrigel) is supposed to allow for an estimation of how many cells will invade through the matrigel. I plated 25,000, 50,000 and 75,000 cells in control inserts (in duplicate) and allowed them to migrate overnight. The control inserts containing 75,000 cells were more than 60% confluent, and so I decided to plate 100,000 cells in the invasion chambers in hopes of increasing the amount of cells that would invade. The cell suspension was prepared in serum free media and 500 µL containing 100,000 cells was added to each chamber as well as a control insert. A volume of 750 µL of media with serum was added to the
bottom of the wells as a chemoattractant. Finally, vehicle and treatments were added to the chambers. Each treatment and control was done in duplicate.

At the end of the treatment time point, the media was aspirated from each of the inserts. A volume of 1 mL of 10% buffered formalin was added to the wells of a 24 well plate. The inserts were placed in the wells containing the formalin and fixed for 5 minutes. Immediately following fixation, the inserts were placed in wells containing a 10% crystal violet solution. The inserts were stained for 5 minutes. Staining was followed by four consecutive washes in distilled water. Next, the membranes within the inserts were “scrubbed” with cotton swabs to remove non-invading cells. Finally, the membranes were removed from the inserts with a scalpel and mounted on slides.

**Results**

The Western blots of MMP family members required a great deal of optimization. Several antibodies were tried and the protocol was adjusted because several of the MMP proteins are secreted. For this reason, media was loaded in the polyacrylamide gel instead of cell lysate (Figure 4.1). The cell lysate was used as a loading control because no housekeeping genes are secreted in the media (Figure 4.1). Once this change was made, the antibody was able to detect protein. I found that Rhodiola treatments did not have an effect on either MMP-3 or MMP-9 expression (Figures 4.2 and 4.3, respectively). Attempts were made to examine MMP-2 expression; however, I was never able to get bands that were good for analysis. The MMP-2 antibody detects two bands, one of which was difficult to resolve.
The scratch wound assay revealed striking results (Figure 4.4). The cells in the vehicle treated well were able to partially recover the wound. It is clear that the cells have been able to make contact with one another and migrate across the cleared area. Conversely, the cells in the Rhodiola treated groups were not able to migrate across the wound as well. In the wells with lower Rhodiola concentrations there were some cells moving into the wound. However, the wells treated with higher concentrations look very much like they did right after the scratch was made. There is no way to quantify the results of this assay; however, it appears as though there is a dose-dependent response to treatment in regards to migration.

As previously mentioned, the matrigel invasion assay required a great deal of optimization. The first time the assay was attempted (Figure 4.5), the membranes were removed from the inserts and then fixed and stained. The stipulation with this procedure is that the scrubbing is not very efficient. I believe that there were non-invading cells still on the membrane when the images were captured and so the results are not completely accurate. For the last two trials, the membranes were left in the inserts during fixation and staining. This method appears to have worked better because you can clearly see the non-invading cells being removed from the membrane during scrubbing. Before the inserts were processed, I captured images because there was a striking difference between the vehicle-treated insert verses the treatments (Figure 4.6). Rhodiola appears to influence invasion, even at low concentrations. Unfortunately, I could not image the mounted slides because the proper microscope and camera were not available.
Discussion

Both the scratch wound assay and the matrigel invasion assay offer strong evidence that *Rhodiola crenulata* was able to influence migration and invasion, respectively. There is a dose-dependent response in the scratch wound assay. The cells treated with lower concentrations of Rhodiola are able to slightly extend into the cleared area whereas those treated with higher concentrations do not do so. Unfortunately, because the matrigel assay protocol took many trials to optimize, I was only able to do the treatments in duplicate. This assay should definitely be repeated, because the preliminary data is very promising. If possible, a comparison to a much less invasive cell line would prove useful for the analysis.

The Western blots were repeated several times, but most of the results could not be analyzed. What the preliminary data shows is that *Rhodiola crenulata* does not have an effect on MMP-3 or MMP-9 protein expression. Other proteins involved in invasion and metastasis were investigated (MMP-2, N-cadherin) but again, results were not good enough for analysis or I could not get the antibody to work.

The results from the aforementioned assays show that Rhodiola is working in some way to influence migration and invasion. My goal was to help uncover the pathway Rhodiola is working on in metastasis. Unfortunately, the Western blots did not help to complete this investigation. Past studies have shown certain compounds to have an effect on proteins involved in invasion and metastasis. Larkins et al. (2006) used an enzyme immunoassay kit to show that COX-2 inhibitors can decrease MMP secretion. Real-time PCR was also done using specific MMP primers and it showed that COX-2 inhibitor
treatment was able to decrease transcript levels of certain MMP family members (Larkins et al. 2006). It would be beneficial to utilize these techniques to further study Rhodiola’s effect on MMP proteins.

It is very possible that Rhodiola simply does not affect MMPs but influences other pathways. A very recent paper by Wang et al. (2007) investigates the cofilin pathway in breast cancer metastasis and invasion. The authors believe that the cofilin pathway is active in motile, invasive cells and that the output of this pathway has an effect on the metastatic potential of mammary tumors (Wang et al. 2007). A review by Christofori (2006) offers a great deal of insight into new thoughts on invasion. The author discusses proteins believed to be involved in metastasis, the role of hypoxia and cells of the immune system and how they modulate tumor progression.

I have provided preliminary data showing that it is possible for Rhodiola to affect a very highly proliferative and invasive cell line. Obviously, there are numerous pathways available for Rhodiola to influence. A more thorough investigation is necessary to shed light on the mechanisms involved; multiple proteins should be examined through a combination of assays (protein/gene expression, activity, etc.).
Figure 4.1 Western immunoblots of 4T1 media and cell lysates. MMP-3 antibody (A), MMP-9 antibody (lower bands) (B), tubulin antibody (C).
Figure 4.2 Quantification of MMP-3 Western blot. Statistics not done; only one blot was able to be analyzed.
Figure 4.3 Quantification of MMP-9 Western blot. Statistics not done; only one blot able to be analyzed.
Figure 4.4 Effect of Rhodiola treatment on wound recovery. Cells were grown to 100% confluency, a wound was created with a pipette tip and treatments were carried out for 24 hours. (A) Before scratch wound, (B) after scratch wound, (C) vehicle treatment, (D) 1:150 Rhodiola, (E) 1:100 Rhodiola, (F) 1:75 Rhodiola, (G) 1:50 Rhodiola, (H) 1:25 Rhodiola.
Figure 4.5 Selected images from matrigel invasion assay. 25,000 cells seeded in invasion chambers and treated with Rhodiola for 24 hours. Membranes fixed with 10% formaldehyde and stained with hematoxylin and mounted on slides. (A) Vehicle treatment, (B) 1:150 Rhodiola, (C) 1:100 Rhodiola and (D) 1:50 Rhodiola.
Figure 4.6 Images of matrigel invasion chambers. 100,000 cells plated and treated with Rhodiola for ~20 hours. (A) Vehicle treated, (B) 1:150 Rhodiola, (C) 1:100 Rhodiola, (D) 1:75 Rhodiola, (E) 1:50 Rhodiola, (F) 1:25 Rhodiola.
CHAPTER 5
RHODIOLA *IN VIVO*

**Introduction**

A study by Aslakson and Miller (1992) found that 4T1s metastasize mostly by a hematogenous route. In 38 out of 58 mice, clonogenic tumor cells were found in the blood whereas only 12 out of 58 mice had tumor cells in the lymph nodes (Aslakson and Miller 1992). When the spread of 4T1 cells was monitored after mammary fat pad injection, 4T1 cells were first detected at day 7 in the lungs and there was 100 percent incidence by day 14. The doubling time was estimated to be 30 hours (Aslakson and Miller 1992). Metastasis to the liver was not as quick. 4T1 cells were first detected at day 21 and it was not until day 28 that all mice had metastasis to this location (Aslakson and Miller 1992).

A second experiment was done and the results were found to be reproducible. In both experiments necropsies were carried out between day 31 and 40. All mice had lung nodules and several had visible liver nodules (Aslakson and Miller 1992). It is important to note that it takes a small amount of 4T1 cells to form a primary tumor. Pulaski and Ostrand-Rosenberg (1998) found that after injecting only 5000 cells subcutaneously into the mammary gland, 100 percent of mice formed primary tumors that were palpable between 11 and 26 days. Increasing the dose of 4T1 cells for injection led to more rapidly forming tumors (Pulaski and Ostrand-Rosenberg 1998).
Our goal was to use the MAESTRO imaging system to track the formation of primary tumors using a fluorescent 4T1 construct. We also wanted to investigate whether or not Rhodiola would have an influence on the formation of primary tumors.

**Materials and Methods**

**Animal work**

*In vivo* experiments were carried out using the 4T1 cell line for a two week imaging project using the Maestro system. A total of 100,000 cells (4T1 construct with mOrange) were injected into the mammary fat pad of BALB/c mice. Treated mice received Rhodiola in their water, which was changed every other day. Mice were anesthetized with Avertin every other day and imaged (Figure 5.1).

**Results**

The sample number (n = 4) was very small for the treatment and control groups but the results were promising. There was a difference in tumor size and weight between the control and treatment groups (Table 5.1). Because the sample number was so small the results are not significant, however, they do offer promise for a larger study using Rhodiola *in vivo*.

**Discussion**

It would be ideal to repeat this study with a larger sample number and also to extend the length of the study. A future study should include both adjuvant and neo-
adjuvant therapy with Rhodiola. After the formation and resection of primary tumors, treatment should continue so that effects on metastasis can be observed and the imaging system can be utilized to track tumor formation.
Figure 5.1 Representative image from MAESTRO imaging study. BALB/c mouse injected with 100,000 4T1 cells (mOrange construct) in the mammary fat pad. Mice treated with Rhodiola or control. Images captured every other day.
Table 5.1 Tumor measurements from control and Rhodiola mice (n=4).

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<td>Rhodiola</td>
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In the year 2007, there will be over 180,000 new breast cancer cases and approximately 41,000 deaths from the disease (NCI). The majority of these cases will involve tumors that are estrogen receptor (ER) positive. These cancers can be treated with selective estrogen receptor modulators (SERMs) or aromatase inhibitors. However, there is a subset of patients who are estrogen receptor negative and therefore cannot benefit from the aforementioned therapies. It is these cases that require new types of treatment especially since ER-negative cancers tend to be more aggressive and have a worse prognosis as compared to ER-positive cancers (Rochefort et al. 2003).

As previously mentioned, it is also believed that COX-2 overexpression leads to a poorer prognosis. Evidence exists that COX-2 is associated with several factors related to cancer including the formation of new blood vessels, higher proliferation and lower apoptosis (Bundred and Barnes 2005). For these reasons, it is also important to regulate COX-2 in order to treat certain breast cancers.

The 4T1 cell line is estrogen receptor negative and expresses COX-2, which makes it the ideal model for studying new therapeutic approaches. As already discussed, a good deal is known about natural compounds, including phenolics, and their influence on cancer. It has been established that phenolics are one of the active ingredients in Rhodiola, however, the exact way in which the extract exerts its effects is unknown. I chose to treat 4T1 cells with Rhodiola in order to see the effects it might have on this
particular cell line. I also wanted to investigate the mechanisms involved in its action, specifically in regards to metastasis.

Through both in vitro and in vivo studies, I have shown that *Rhodiola crenulata* is effective in inducing death and inhibiting both migration and invasion in a highly metastatic ER-negative mammary carcinoma cell line. Because Rhodiola is a natural compound, and the concentrations used are physiologically relevant, it may be useful for both chemoprevention and treatment.

Unfortunately, my studies did not lead to any novel findings in regards to how Rhodiola affects the metastatic potential of 4T1 cells. I chose to study the MMP family of proteins because of their broad implication in cancer invasion and metastasis, but the preliminary results are not very striking. This being said, my study is a very narrow one, focusing specifically on one class of proteins, when there are a multitude of pathways and families of proteins to be investigated. As mentioned throughout, there are many ways this study can be broadened so that more detailed results can be obtained.
REFERENCES


