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Effects of Phytochemicals from Rhodiola crenulata on Highly Invasive Breast Cancer Cell Lines and Embryonic Models of Migration

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EFFECTS OF PHYTOCHEMICALS FROM *RHODIOLA CRENULATA* ON HIGHLY
INVASIVE BREAST CANCER CELL LINES AND EMBRYONIC MODELS OF
MIGRATION

A Dissertation Presented

by

ADARIS RODRÍGUEZ-CORTÉS

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
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EFFECTS OF PHYTOCHEMICALS FROM RHODIOLA CRENULATA ON HIGHLY INVASIVE BREAST CANCER CELL LINES AND EMBRYONIC MODELS OF MIGRATION

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DEDICATION

I dedicate this work to my mother, Mini, and grandmother, Mamá Tinita, who raised me with more love than anyone would think possible and with a sense of responsibility that will never abandon me. To my dad, who though not present during my upbringing I am believe has always been present in spirit and watches over me. To my beloved aunts, who have been an integral part of my education and my upbringing, have always cherished me with their love and made huge sacrifices to propel our family, give us the gift of education, instill in us moral principles and values, and for the dedication and love you put into everything you do. To my beloved fiancé, future husband, and best friend, David. You make me smile every day with your presence, keep me grounded with your honesty, fill me with happiness and your love. Your passion for life and charisma are infatuating and keep me going every day!
EPIGRAPH

"Always move forward. Never walk back, not even to propel yourself, remember this sweetheart." - Uncle Iche

“Siempre pa' lante. Pa' tras ni pa' coger impulso. Pa' tras nunca, recuérdalo, Negrita.” - Tio Iche

“You have talents that you cannot let go to waste. If you have wings, and you do, fly and fly high; your imagination and you God should be your only limit.” –Mom

“Tú tienes unos talentos que no puedes desperdiciar. Si tienes alas, y las tienes vuelo, y vuelo alto; que tu imaginación y tu Dios sea tu único límite.” –Mami
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ABSTRACT

EFFECTS OF PHYTOCHEMICALS FROM RHODIOLA CRENULATA ON HIGHLY INVASIVE BREAST CANCER CELL LINES AND EMBRYONIC MODELS OF MIGRATION

SEPTEMBER 2013

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Directed by: Professor Sallie Smith Schneider

The root of the Tibetan plant Rhodiola crenulata is part of eastern traditional medicine. Studies have suggested that members of the Rhodiola genus display anticancer properties. In this study we examine the effect of R. crenulata in a cellular model of invasive breast cancer, this disease being the second cause of cancer death among women in the US. Deregulation of the Wnt/β-catenin pathway has been frequently observed in breast cancers and appears to have a key role in the transformation of benign cells to a malignant form. Although mutations of the Wnt growth factor are rarely observed in cancer, the Wnt signaling pathway is often up-regulated by either mutations that result in stabilization of β-catenin or by hypermethylation and subsequent loss of expression of Wnt signaling antagonists like secreted Frizzled-Related Protein 1 (SFRP1) (Hanahan and Weinberg 2000; Miyoshi, Rosner et al. 2002; Reya and Clevers 2005; Suzuki, Toyota et al. 2008) (Hanahan and Weinberg 2000; Miyoshi, Rosner et al. 2002; Reya and Clevers 2005; Suzuki, Toyota et al. 2008) (Hanahan and Weinberg 2000; Miyoshi, Rosner et al. 2002; Reya and Clevers 2005; Suzuki, Toyota et al. 2008). We used an engineered cell line in which SFRP1 expression has been knocked down. These cells were derived from 76N Tert cell line, an immortalized human mammary epithelium cell line. The resulting 76N Tert-siSFRP1 cells display a mesenchymal-like phenotype, invasive behavior and are more resistant to apoptosis triggered by anchorage independent conditions, or anoikis. Additionally we used a highly invasive estrogen receptor negative (ER-), progesterone receptor negative (PR-), Her2/neu negative (triple negative) breast cancer cells line MDA-MB-231. Treatment of MDA-MB-231 and 76N Tert-siSFRP1 cells with an extract of R. crenulata inhibited migration and invasion of both cell types, as compared to untreated cells. Furthermore, R. crenulata sensitizes cells to anoikis but does not increase γ-irradiation induced cell death. We provide evidence that death induced by R. crenulata does not occur through the inhibition of an epithelial-to-mesenchymal transition (EMT). Taken together, our initial results suggest R. crenulata as a potential therapeutic agent for breast cancer patients with mutations in the Wnt/β-Catenin signaling pathway.

Additionally, we present evidence that R. crenulata exerts its anti-metastasis effect by inhibiting cell migration and increasing cell attachment to a substrate. We demonstrate that this effect occurs by R. crenulata’s modulation of the Rho GTPase effector ROCK1.
We further show evidence that *R. crenulata*’s effect on ROCK is not limited to cancer cells (*in vitro*), but also affects isolated and intact embryonic tissues. We discovered that treatment of *X. laevis* embryos with *R. crenulata* can cause a *spina bifida* phenotype, suggesting (1) that compounds in *R. crenulata* may prove detrimental to a developing embryo, and (2) the active compounds in *R. crenulata* may prove useful in the study of developmental anomalies that lead to conditions such as *spina bifida*. More importantly, our results suggest that pregnant women should avoid taking *R. crenulata*-containing supplements during pregnancy. Compounds in *R. crenulata* may be contraindicative to the pregnancy and cause injury to a developing fetus. The information provided may help health providers offer better advice on natural supplements to expecting mothers.

We also characterized and identified multiple *R. crenulata* compounds and report predicted protein targets for these compounds. Finally, we provide evidence that *R. crenulata* affects cancer cell metabolism and suggest potential protein targets of the chemical components of this extract. This study provides new information that will help dissect the mechanisms of action of the *R. crenulata* compounds and possible synergies amongst them.
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CHAPTER 1

BREAST CANCER, INSIGHT INTO ITS MOLECULAR CAUSES, ITS CURRENT THERAPIES AND CURRENT TREATMENTS

Breast Cancer

Breast cancer is the second leading cause of cancer-related death among women in the United States of America (U.S.A.) and the main cause of physical illness-related deaths in high-income countries (AbouZahr, De Zoysa, & Garcia Moreno, 2010). The American Cancer Society (ACS) has estimated a total of 232,620 newly reported breast cancer cases for 2011 in the U.S. alone, of which 230,480 will be females and 2,140 males (Breast Cancer: Facts & Figures 2011-2012, 2012a; Desantis, Siegel, Bandi, & Jemal, 2011). The ACS has also estimated that during the current year, 2011, a total of 39,970 deaths will occur due to breast cancer. Besides the high incidence of this disease and the psychological burden that accompanies patients and caregivers, breast cancer, one of the five most common cancers diagnosed in the USA, also brings about an immense financial toll. The NCI reported that in 2006 breast cancer alone cost the American public 13.89 billion dollars (Bensaad, Tsuruta et al.) in cancer care and 12.1 billion dollars (Bensaad, Tsuruta et al.) in losses in time and productivity (Hardison, 2010). The majority of the expenditures are invested in the treatment of survivors in the continuing care phase of treatment (Nance-Nash, 2011). The continuing phase of treatment consists of adjuvant therapy, routine physical examinations and imaging tests to ensure that, after surgical removal of a tumor, no cancer cells remain in the patient. The continuous care phase is
critical for a full recovery, the prevention of relapse and further complications. Although a localized breast cancer can be removed by surgery, the main cause of breast cancer-related deaths is a result of invasive mammary cancers that metastasize and invade other body organs and tissues, including the lymph node, lungs, bone, liver and most other tissues (DiSibio & French, 2008).

Despite the present day breast cancer awareness movement and the many advances in the treatment of mammary carcinomas, breast cancer is an ancient disease indifferent of ethnicity or social status. The road to current therapies and the understanding of this disease came about only recently, during the nineteenth century. Nevertheless, descriptions of the disease and approaches taken to treat it were documented several millennia ago. The report was written at around 3,000 BC by the Egyptian physician Imhotep, in The Edwin Smith Surgical Papyrus (Donegan, 2006). Imhotep described male patients with breast overgrowths, descriptions that have a striking resemblance to modern day breast tumors. Imhotep noted that these overgrowths were untreatable. Other cases were documented in the ancient world by Herodotus (484-425 BC), Hippocrates (460-375 BC) and physicians of the school of Alexandria during the Hellenistic period, such as Leonides (also known as Leonidas, 100 – 180 AD) who first described in detail the surgical removal of breast cancers via excision of localized tumors and resection around the tumor. Leonides' technique involved alternate cutting and cauterizing the wound and ill tissue. Leonides also reported the spread of breast cancers to the axilla and the occurrence of breast cancer mainly in women but rarely in men. Reports such as that of Leonides continued to appear up to the Middle Ages when science was contested. It was not until the Renaissance, the 16th century that the advent of anatomy and advances
in science, such as the development of the microscope, allowed scientists to gain better understanding of nature and of the components that make the tissues of living organisms, cells. Once it was established that all tissues of complex organisms are made up of cells, the advancements in biology and medicine advanced in leaps, giving rise to modern day medicine and to a better understanding of disease.

The female breast organ is heterogeneous and contains distinct tissues and structures. It is mainly composed of lobules (milk producing glands), ducts (which connect the lobules to the nipple) and the surrounding stromal tissue (connective tissue and fatty tissue) (Figure 1). These tissues undergo cycles of morphological alterations throughout an individual’s lifetime. The alterations initiate during puberty in response to hormones when mammary ducts, composed of mammary epithelial cells, extend and branch throughout the breast fat pad. These highly branched ducts form what is known as the “ductal tree” (Figure 2a). A second alteration to the mammary gland takes place after conception. During pregnancy the ductal tree expands via a process that requires massive cellular proliferation (Figure 2b). The third alteration occurs during the late stages of pregnancy and following the birth of the progeny, when milk production ensues. During lactation epithelial cells of the ductal tree differentiate and initiate the production of milk (Figure 2c). After weaning, the mammary gland undergoes a process known as involution, which entails extensive apoptosis, or programmed cell death, of the ductal tree cells, returning the tissue to a non-pregnant-like state (Figure 2d). Because the tissue has completed morphological changes of a full term pregnancy, we refer to it parous (having given birth) mammary gland. The mammary gland development cycle repeats
during the reproductive life of female mammals, ensuring that the mammary organ is properly equipped for each gestational stage.

The complex dynamics involved in the development of the mammary gland are elegantly tuned by an array of molecular pathways. These pathways function as molecular checkpoints and they orchestrate the expansion, growth, differentiation or death of cells of the mammary gland and surrounding tissue. Deregulation of these molecular checkpoints results in a compromised mammary development and, if not corrected can lead to the uncontrolled growth and behavior of the mammary tissue. Often the deregulated development of the mammary gland can lead to uncontrolled growth of mammary cells and this can lead to and result in breast cancer. Figure 3 depicts the stages of breast cancer.

Diverse biological and environmental factors contribute to the high incidence of breast cancer. These factors include an individual’s gender, age, genetic background, familial medical history, personal history of breast cancer, race and ethnicity, density of the individual’s breast tissue, expression profile of steroid receptors, histological grade of a lesion, other benign breast conditions, previous chest exposure to radiation, and lifestyle-related factors such as contraceptive use, postmenopausal hormone treatment, alcohol consumption, being overweight or obese, levels of physical activity, and the status of menses, estrous, parity, and breast-feeding. Table 1 shows the relative risk of factors that increase the risk of breast cancer in women. Additionally complications can ensue, including the recurrence of the cancer following remission and the metastasis of highly invasive cancer types. To better treat breast cancer and improve patient prognosis, early detection, diagnosis and an aggressive treatment regime are indispensable. Given the
heterogeneous risks that contribute to developing breast cancer and the differences amongst diseased individuals, much research is currently underway to develop new therapies that improve current treatments and that possess a more universal effect against different types of cancers.

**Pathways Involved in Breast Cancer**

Scientists have put a large amount of effort into understanding the behavior of cancers and how to treat them effectively. In the year 2000 Hanahan and Weinberg suggested that cancer cells acquire six traits that, collectively, give rise to a deregulation of their cell cycles and physiology and ultimately result in their malignant growth (Hanahan & Weinberg, 2000). These traits were identified as follows: self-sufficiency in growth signals (production of growth factors by the cancer cells), insensitivity to anti-growth signals, evasion or resistance to apoptosis, unlimited replication potential, sustained angiogenesis in formed tumors, and the capability to invade and metastasize to other tissues (Hanahan & Weinberg, 2000). More recently Gatza and colleagues have developed a pathway-based classification of human breast cancer. The group studied the transcriptional profiles of a large collection of breast tumor gene expression data and successfully identified 18 unique molecular pathway signatures that encompassed all samples. These signatures successfully group tumors into subsets that exhibit similar clinical and biological properties, similar patterns of pathway activity and possess similar chromosomal alterations (Gatza et al., 2010). The signature pathways that were identified and validated by this research group were as follows: Akt, β-catenin, EGFR, E2F1, ER, Her2, IFNα, IFNγ, Myc, p53, p63, PI3K, PR, Ras, Src, STAT3, TGFβ, and TNFα, all
well known oncogenic pathways (Gatza et al., 2010). These signature pathways are known to regulate the processes necessary for the malignant transformation and behavior of cancer cells. In more aggressive cancers, one can often find many cells exhibiting the traits of a process known as the Epithelial to Mesenchymal transition (EMT). During this transition epithelial cells undergo morphological and physiological changes. EMT cells become stromal in shape and highly motile. EMT cells will often display stem cell-like traits, or a lack of cell differentiation. The EMT cancer cells gain the capability to produce more cancer cells. In other words they become progenitors that can give rise to cells identical to or different from the parent cells. EMT cells also exhibit increased glycolytic and fermentation rates (a phenomenon known as the Warburg effect), increased motility and invasiveness, the capability to digest the basement membrane (a usually thin layer of extracellular matrix proteins that lines the epithelial layer) and migrate to distant sites, resistance to programmed cell death, also known as apoptosis, insensitivity to cell-cell contact inhibition and loss of cell polarity and hence, subsequent loss of directionality. The study by Gatza, et al. (2011) promises to provide a useful aid in the design of the course of treatment for specific cancer types that possess any or a combination of the processes described above. Nevertheless, much research is still ongoing to identify compounds useful to treat cancers that bear currently untreatable signature pathways. Additionally, much effort is being invested into identifying compounds that will halt and kill cancer cells in an organism while also not affecting or even protecting normal cells and, hence, tissues. In summary, many advances have been made that will allow the scientific community to better employ current therapies and to develop new, more effective treatments.
Current Breast Cancer Treatments

Since the 1970s, breast cancer awareness has increased in the U.S. and throughout the world. Right up to the 1950’s any disease or condition of the breast was considered a taboo subject and was, hence, rarely discussed. This mentality and the prevalence of Galen’s teachings (see Figure 4), who considered that breast cancer could only be cured by complete excision of the breast or radical mastectomy (Donegan, 2006), prevented women from seeking help if they found an abnormality in their breasts. In the late 1800s and early 1900s the use of radiotherapy to treat cancer had developed and was given as an adjuvant treatment to patients with breast cancer. Nevertheless, the fear to speak about the subject with such social impact kept early detection and early treatment of a malignancy at bay. Three events caused great scandal as they challenged the social taboos of the time. In 1953, Hugh Hefner published the first edition of Playboy magazine (Olson, 2005), changing the perception of the female breast from a taboo (with the invention of the feeding bottle in the 19th century the need for wet nurses waned and the breast became mostly a symbol of eroticism (Stevens, et al. 2009)) to a marketable product. This novel perception also led to the conviction of many women that it was necessary to evaluate their own breasts for size, shape and firmness. Thus a new market was born, but it also led to a new view on the feminine physique that, albeit an aesthetic view, raised awareness in women of the female breast. The second event happened in the city of Chicago in 1956 when two mothers, Marian Thompson and Mary White received disgusted looks when they sat on a park bench to nurse their infants. Two weeks later the pair founded the La Leche League, via which they educated women on the benefits of breast feeding and how to nurse their infants modestly in public scenarios. Leaguers
focused on emphasizing the view of the breasts as functional entities rather than sexual objects to be shunned by prudery. It was also the League who began to emphasize epidemiological evidence that women who breastfed had lower incidence of breast carcinoma, possibly founding the first campaign of breast cancer awareness. A huge increase in screening and diagnosis of breast cancer in the occurred in the 1970s, and was termed as the “Betty Ford Blip”. On September 28th, 1974, the First Lady of the USA, Elizabeth Ann Ford, also known as Betty Ford, underwent a mastectomy. The results of this procedure resulted in her diagnosis of breast cancer. Her decision to be open about her condition and discussing it in public raised the visibility of breast cancer, a disease that many Americans would not talk about until then. The public announcement of Betty Ford’s condition led an immense number of women to perform self-exams. These self-examinations resulted in an increase in the detection and diagnosis of breast anomalies and hence an increase in the number of reported breast cancer cases. This awareness has driven an immense investment into research and technology to treat this disease as well as other cancers. As we learned more about the biology of cancer, what processes drive its seemingly uncontrollable growth and aggressive nature, and the molecules involved in its intrinsic signaling pathways, we developed tools to manipulate cancer cells and their innate processes. Out of these findings we developed an array of treatments for specific breast cancer types. A list of the treatments for breast cancer that have been developed to date is given in Table 2. When available, the table includes a column indicating the range of years since the discovery of a drug to its approval by the Food and Drug Administration (FDA). Many of these treatments, as will be discussed in the following chapter, are derived from plants and microorganisms and such biologically derived
compounds have proven highly effective in the treatment of mammary and many other types of cancers.

Figure 1. Anatomy of the Breast. Left: Diagram of the tissues of a normal breast with enlargement of the lobular structures. Top right: Diagram of the tissues of a normal breast with emphasis on the relative position of the breast with respect to the torso. (A) Ducts, (B) Lobules, (C) Dilated section of duct to hold milk, (D) Nipple, (E) Fat, (F) Pectoralis major muscle, and (G) Chest wall/rib cage. Bottom right: (A) Normal duct cells, (B) Basement membrane, and (C) Lumen (center of duct). (Images were taken from (Dna, 2010 and http://www.breastcancer.org/pictures/types/dcis/dcis_range.jsp)
Figure 2. Stages of Development of the Mammary Gland in Mice. Schematic representations (A-D Left) and murine mammary gland whole mounts (A-D Right) representing of the different stages of mammary gland development. A simple duct is present within the mammary fat pad at birth and it grows at the same rate as the individual’s growth until the individual reaches puberty. (A) During puberty, the cycles of estrogen and progesterone production promote and accelerates ductal outgrowth, and terminal end buds (TEBs) appear at the ductal tips. The TEBs is where the highest level of cell division takes place. In the mature virgin individual, the entire fat pad is filled with regularly primary and secondary ducts. (B) During each estrous cycle side branches are formed and they disappear as the estrous cycle ends. (C) When pregnancy begins hormonal changes, including the release of prolactin, placental lactogens and progesterone, increase cell proliferation. It is then that alveolar buds are formed. The alveolar buds grow and differentiate into milk-secreting alveoli at the end of pregnancy. During lactation, alveoli are fully matured and the luminal cells synthesize and secrete milk components into the lumen. (D) Once lactation ceases a massive event of apoptosis termed involution takes place. This mass apoptosis returns the mammary gland to a pre-pregnancy state. (Bissell, Radisky, Rizki, & W, 2002; Hennighausen & Robinson, 2005)
**Table 1. Factors That Increase the Risk for Breast Cancer in Women**

<table>
<thead>
<tr>
<th>Relative Risk</th>
<th>Factor</th>
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<tbody>
<tr>
<td>&gt;4.0</td>
<td>- Age (65+ vs. &lt;65 years, although risk increases across all ages until age 80)</td>
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<tr>
<td></td>
<td>- Biopsy-confirmed atypical hyperplasia</td>
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<td></td>
<td>- Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2)</td>
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<td></td>
<td>- Mammographically dense breasts</td>
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<td></td>
<td>- Personal history of breast cancer</td>
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<tr>
<td>2.1-4.0</td>
<td>- High endogenous estrogen or testosterone levels</td>
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<td></td>
<td>- High bone density (postmenopausal)</td>
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<tr>
<td></td>
<td>- High-dose radiation to chest</td>
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<tr>
<td></td>
<td>- Two first-degree relatives with breast cancer</td>
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<tr>
<td>1.1-2.0</td>
<td>- Alcohol consumption</td>
</tr>
<tr>
<td></td>
<td>- Ashkenazi Jewish heritage</td>
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<tr>
<td></td>
<td>- Early menarche (&lt;12 years)</td>
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<td></td>
<td>- Height (tall)</td>
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<td></td>
<td>- High socioeconomic status</td>
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<td></td>
<td>- Late age at first full-term pregnancy (&gt;30 years)</td>
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<td></td>
<td>- Late menopause (&gt;55 years)</td>
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<tr>
<td></td>
<td>- Never breastfed a child</td>
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<tr>
<td></td>
<td>- No full-term pregnancies</td>
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<td></td>
<td>- Obesity (postmenopausal)/adult weight gain</td>
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<td></td>
<td>- One first-degree relative with breast cancer</td>
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<td></td>
<td>- Personal history of endometrium, ovary, or colon cancer</td>
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<tr>
<td></td>
<td>- Recent and long-term use of menopausal hormone therapy containing estrogen and progestin</td>
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<tr>
<td></td>
<td>- Recent oral contraceptive use</td>
</tr>
</tbody>
</table>

**Table 1. Factors that Increase the Risk for Breast Cancer in Women.** The table lists known breast cancer risk factors and groups them under Non-modifiable risks (top and middle sections) and Modifiable risks (bottom section). Non-modifiable risks are those that involve genetic predisposition and a previous medical history of cancer. The Modifiable risks encompass lifestyle-related habits or results of habits that affect the individual’s fitness or overall health. Other risks, not included in this table, involve financial status and accessibility to health centers and adequate treatments. (Table was taken from *Breast Cancer: Facts & Figures 2011-2012*, 2012b)
Table 2. Breast cancer treatments, dates of discovery, uses and common side effects.

<table>
<thead>
<tr>
<th>Name</th>
<th>Alternate Name(s)</th>
<th>Scientist(s)</th>
<th>Type of Chemotherapy</th>
<th>Use(s)</th>
<th>Common Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoxan</td>
<td></td>
<td></td>
<td>Alkylating agent</td>
<td>Used to treat a variety of cancers, including breast cancer. Alkylating agents modify the bases of DNA. The alterations interfere with DNA replication and RNA transcription, they lead to mutations and cell damage. Therefore, rapidly dividing cells, such as cancer cells, accumulate mutations more rapidly and are damaged faster than normally dividing cells.</td>
<td>Myelosuppression - damage to the bone marrow that eventually leads to anemia, stomatitis - irritation of the mucous membranes of the mouth, alopecia - falling of the hair, decreased fertility, nausea, vomiting, anorexia, upset stomach and diarrhea.</td>
</tr>
<tr>
<td>Halotestin</td>
<td></td>
<td></td>
<td>Anabolic steroid hormonal therapy</td>
<td>A synthetic testosterone that is used to treat women with hormone-dependent breast cancer, especially recurrent estrogen-receptor positive breast cancer.</td>
<td>Nausea, vomiting, headache, skin color changes, increased or decreased sexual interest, oily skin, hair loss, acne, amenorrhea, virilization, inhibition of gonadotropin secretion, gynecomastia, fluid retention and electrolyte disturbances, hirsutism, seborrhea, jaundice, hepatocellular neoplasms, peliosis hepatitis, clotting defects, polycythemia, decreased libido, headache, anxiety, parasthesia, and skin hypersensitivity or allergies.</td>
</tr>
<tr>
<td>Doxil, daunorubicin, Eilence, mitoxantrone, Adriamycin</td>
<td></td>
<td></td>
<td>Anthracycline</td>
<td>Anthracyclines are very effective anticancer treatments. They are effective against more types of cancer than any other class of chemotherapeutic agents. They act by inhibiting topoisomerase II and creating free oxygen radicals (reactive oxygen species, ROS) that damaged the cell's DNA. Anthracyclones are compounds, derivatives of antarouquinone, that have antineoplastic properties. They are usually used to treat metastatic breast cancer, acute myeloid leukemia, and non-Hodgkin's lymphoma.</td>
<td>Pain along the site where the medication was given, nausea or vomiting, low blood counts, mouth sores, and hair loss.</td>
</tr>
<tr>
<td>Name</td>
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<tr>
<td>Adrucil</td>
<td>Fluouracil, 5-FU, &quot;5 Feet under&quot;</td>
<td>Richard Lewenhsohn and Sidney Farber</td>
<td>Antimetabolite</td>
<td>Antimetabolites inhibit the processing of a small molecule that is usually processed in a metabolic pathway (metabolite). 4-aminofolic acid was used to treat children with acute leukemia. Years later the FDA approved methotrexate which is less toxic than 4-aminofolic acid. Methotrexate is used to treat certain types of cancer including cancers that begin in the tissues that form around a fertilized egg in the uterus, breast cancer, lung cancer, certain cancers of the head and neck, certain types of lymphoma, and, in combination with prednisone, for the treatment of acute lymphoblastic leukemia (ALL).</td>
<td>Each antimetabolite has a distinct set of side effects. The following are the side effects of treatment with 5-FU: Thinned or brittle hair, headache, weakness or achiness, drying or darkening of the skin or nails, sensitivity of skin to sunlight, blistering skin or acne, loss of appetite or weight, tingling in the hands or feet, diarrhea, nausea, vomiting, mouth sores, vision or eye problems, taste changes, metallic taste in mouth during infusion and low white blood cell count.</td>
</tr>
<tr>
<td>Gemzar</td>
<td>Armethoterpin, Mexaile, Folex, Rheumatrex Xeloda</td>
<td></td>
<td>Antimetabolite</td>
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<tr>
<td>Mitomycin, MTC</td>
<td></td>
<td></td>
<td>Antitumor antibiotic chemotherapy</td>
<td>An arizidine-containing antibiotic that as activity against tumor cells. Mainly works by cross-linking DNA</td>
<td>Hair loss, loss of appetite, nausea and vomiting, headache, blurring of vision, confusion, drowsiness, syncope, fatigue, edema, thrombophlebitis, hematemesis, diarrhea and pain</td>
</tr>
<tr>
<td>Aredia</td>
<td></td>
<td></td>
<td>Biphosphate</td>
<td>Prevents progression of bone metastasis originating from breast cancer. It also prevents bone deterioration deterioration and the resulting calcium release from the skeletal system, thus maintaining blood calcium levels at bay.</td>
<td>Heartburn, difficult or painful swallowing, chest pain, bone, joint or muscle pain, mental or mood changes, stomach pain, fever or sore throat, and upset stomach or diarrhea.</td>
</tr>
<tr>
<td>Zometa</td>
<td></td>
<td></td>
<td>Biphosphate</td>
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<tr>
<td>Ixempra</td>
<td>Epitholone microtubule stabilization</td>
<td></td>
<td>Epitholone microtubule stabilization</td>
<td>Epitholones act similarly but not identically to paclitaxel compounds on mitotic spindles. They stabilize the spindle and prevent cells from dividing. They effectively treat paclitaxel-resistant cancers.</td>
<td>Tired feeling, joint or muscle pain, hair loss, nausea, vomiting, stomach pain, loss of appetite, diarrhea or constipation, or white patches or sores inside the mouth or on the lips.</td>
</tr>
</tbody>
</table>

15
<table>
<thead>
<tr>
<th>Name</th>
<th>Alternate Name(s)</th>
<th>Scientist(s)</th>
<th>Type of Chemotherapy</th>
<th>Use(s)</th>
<th>Common Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faslodex</td>
<td></td>
<td></td>
<td>Estrogen receptor</td>
<td>Downregulates the expression of the estrogen receptor and triggers its degradation preventing the receptor's function</td>
<td>Pain at the injection site, nausea, bone pain, fatigue, pain in the extremities, hot flash, anorexia, asthenia (lack or loss of strength and energy), musculoskeletal pain, cough, dyspnea (shortness of breath), and constipation.</td>
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<tr>
<td></td>
<td></td>
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<td>downregulator (ERD)</td>
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<td>Tykerb</td>
<td></td>
<td></td>
<td>HER-2 Inhibitor</td>
<td>Small-molecule inhibitor of HER-2. It is used in advanced and metastatic breast cancer patients, especially, those who have been already treated with Herceptin</td>
<td>Uneven heart rate, extreme dizziness or fainting, severe diarrhea, dry cough, feeling short of breath, white patches or sores inside the mouth or lips, nausea, stomach pain, low fever, and loss of appetite.</td>
</tr>
<tr>
<td>Lupron, Leuprolarin, Leuprolide acetate</td>
<td>Lupron, Leuprolarin, Leuprolide acetate</td>
<td>Leuprolide acetate</td>
<td>Leuprolide acetate</td>
<td>Lupron is a gonadotropin-releasing hormone (GnRH) analog that acts as an agonist of the GnRH receptors in the pituitary gland and the eventual decrease in production of estrogen and testosterone in both sexes. It is an effective treatment of against estrogen and testosterone responsive tumors. Trelstar and Goserelin act as agonist and superagonists, respectively. They act by overstimulating the pituitary gland such that the pituitary decreases the amount of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) that it releases. Both compounds also have the end effect of lowering estrogen and testosterone levels and hence are used to treat hormone-responsive cancers.</td>
<td>Mild burning, pain, or bruising at the injection site, hot flashes, increased sweating, night sweats, tiredness, headache, upset stomach, breast changes, acne, joint or muscle aches, trouble sleeping, reduced sexual interest, vaginal discomfort or dryness, vaginal bleeding, swelling of the ankles or feet, increased urination at night, and dizziness.</td>
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<tr>
<td>Name</td>
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<tr>
<td>Taxol, Abraxane</td>
<td>National Cancer Institute-United States Department of Agriculture (NCI-USDA), Arthur Barclay; Monroe E. Wall and Mansukh C. Wani; Susan Howitz</td>
<td>Mitotic spindle stabilizer</td>
<td>Used in the treatment of ovarian cancer, breast cancer, non-small-cell lung carcinoma, and Kaposi's sarcoma. Taxol stabilizes microtubules during mitosis, preventing cell division and inducing apoptosis. Taxol was originally isolated from the bark of the Pacific yew tree (Taxus brevifolia) and is currently semi-synthesized from 10-deacetylbaetatin (10-DAB) from Taxus baccata, the English yew plant.</td>
<td>Pain, swelling, redness, or skin color changes at the medicine injection site, joint or muscle pain, mild nausea, vomiting, diarrhea and hair loss. More serious side effects are slow heart rate, seizure (black-out or convulsions), pale skin, easy bruising or bleeding, unusual weakness, fever, chills, body aches, flu symptoms, white patches or sores inside the mouth or on the lips, numbness, tingling, or burning pain in the hands and feet, increased blood flow.</td>
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<td>Taxotere</td>
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<tr>
<td>Paraplatin</td>
<td></td>
<td></td>
<td>Platinum-based</td>
<td>Used in the treatment of nonmetastatic and metastatic testicular cancer, ovarian tumors and bladder cancers.</td>
<td>Nausea, vomiting, kidney toxicity blood test abnormalities, low white blood cell count, low red blood cells (anemia), peripheral neuropathy, high frequency hearing loss, loss of appetite, taste changes, metallic taste, increases in liver function, hair loss, and decreased fertility.</td>
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<tr>
<td>Catatonic (cell killing) platinum, Platinol, cis-dichloroamino-platinum</td>
<td>Barnett; Rosenberg</td>
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</tbody>
</table>
## Breast cancer treatments, dates of discovery, uses and common side effects.

<table>
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<tr>
<th>Name</th>
<th>Alternate Name(s)</th>
<th>Scientist(s)</th>
<th>Type of Chemotherapy</th>
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<th>Common Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Xgeva, Prolia</strong></td>
<td></td>
<td>RANKL inhibitor</td>
<td>Human monoclonal antibody that specifically targets the Receptor activator of nuclear factor Kappa B Ligand (RANKL). RANKL promotes bone loss due to osteoporosis or bone metastasis originating from breast tumors. RANKL is also necessary for metastasis of breast cancer to the bone. Therefore, inhibition of RANKL both prevents bone mass reduction due to osteoporosis or already present bone metastasis, but it also prevents metastasis from a breast tumor to the bone.</td>
<td>Tiredness, weakness, headache, diarrhea, and nausea. More serious side effects are low levels of calcium in the blood, muscle spasms or cramps, mental or mood changes (such as irritability or confusion), numbness or tingling (especially around the lips or mouth or between fingers or toes), seizures.</td>
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<td><strong>Evista</strong></td>
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<td>In 1978 it was approved by the FDA to treat estrogen receptor positive (ER+) metastatic breast cancer. Tamoxifen is a partial ER antagonist that also shows some partial agonist activity in selected organs. It is therefore considered a selective estrogen receptor modulator (SERM) that induces ovulation in female humans. Its long term use results in control of the sexual cycle and limits the growth of breast cancers. It is now used for all stages of ER+ breast cancer and as a risk-reduction medicine.</td>
<td>Common side effects include bone pain, constipation, coughing, hot flashes, muscle pain, nausea, tiredness, vaginal discharge, and weight loss.</td>
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<td><strong>Fareston</strong></td>
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<td>Name</td>
<td>Alternate Name(s)</td>
<td>Scientist(s)</td>
<td>Type of Therapy</td>
<td>Use(s)</td>
<td>Common Side Effects</td>
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<tr>
<td>Herceptin, humanized chMAb 4D5</td>
<td>Axel Ulrich, Dennis J. Slamon, Wendy Levin; Genentech scientists Paul Carter and colleagues</td>
<td>Targeted therapy, monoclonal antibody</td>
<td>Used for the treatment of Human Epidermal Growth Factor Factor Receptor-2 (HER-2; also known as neu) positive (HER-2/neu+) breast cancer tumors. It works by targeting, binding to and inhibiting the proliferative signaling function of HER-2 in cancer cells. Avastin works by targeting and inhibiting the VEGF receptor, hence preventing vascularization of a tumor.</td>
<td>Herceptin: Fever, chills, muscle aches and nausea. A more serious side effect is damage of the heart.</td>
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<tr>
<td>Bevacizumab, Humanized monoclonal antibody against vascular endothelial growth factor (VEGF) receptor</td>
<td></td>
<td>Targeted therapy, monoclonal antibody</td>
<td>Avastin: Changes in taste, constipation, diarrhea, dizziness, dry mouth, dry skin, hair loss, headache, increased thirst, indigestion, loss of appetite, minor nose bleeds, mouth pain or sores, muscle pain, nausea, swelling or redness at the injection site, sluggishness, stuffy or runny nose, tiredness, voice changes, vomiting, weakness, and weight loss. More serious side effects include severe allergic reactions (hives, itching, difficulty breathing, tightness in the chest, swelling of the mouth, face, lips, or tongue), black, tarry stools, blood in the urine, calf pain or tenderness, chest pain, confusion, cough, coughing or choking while eating, coughing up or vomiting blood, dark urine, difficulty or painful urination, fainting, fever, chills or persistent sore throat, frequent or urgent urination, loss of coordination, numbness of the arms or legs, one-sided weakness, peeling of skin, seizure, severe or persistent headache, severe or persistent stomach pain, constipation, nausea or vomiting, severe or persistent weakness, shortness of breath, skin discoloration, irritation, or lesions, speech changes, sudden, severe dizziness, swelling, discoloration, or pain in the legs, swelling of the hands, ankles, or feet, trouble swallowing, unusual bruising or bleeding, unusual or severe sweating.</td>
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<tr>
<td>Eribulin</td>
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<td></td>
<td>Tubulin Inhibitor</td>
<td>Eribulin is used to treat patients with metastatic breast cancer who have received at least two prior chemotherapy regimens for late-stage disease, including both anthracycline- and -taxane-based chemotherapies. Eribulin binds to a small number of high affinity sites at the plus ends of existing microtubules and it exerts its anticancer effects by inducing a prolonged mitotic blockade which eventually results in apoptosis.</td>
<td>Bone pain; constipation; dizziness; hair loss; headache; joint or muscle aches; loss of appetite; mild weight loss; nausea; tiredness; weakness. More severe side effects are severe allergic reactions (rash; hives; itching; difficulty breathing; tightness in the chest; swelling of the mouth, face, lips, or tongue); back pain; burning, numbness, or tingling in the hands or feet; depression; irregular heartbeat; mouth or tongue sores or swelling; muscle pain, weakness, or cramps; severe or persistent tiredness or weakness; shortness of breath; swelling of the hands, ankles, or feet; symptoms of infection (i.e. fever, chills, cough, sore throat, increased or painful urination); unusual bruising or bleeding.</td>
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<tr>
<td>Exemestane, forstenstane, and atamestrane</td>
<td></td>
<td></td>
<td>Type I Aromatase Inhibitor</td>
<td>Type I aromatase inhibitors bind to the aromatase enzymes irreversibly effectively inactivating the enzymes. Amino glutethimide was the first aromatase inhibitor marketed (in 1981) for the treatment of breast cancer.</td>
<td>Risk for developing osteoporosis and joint disorders, i.e. arthritis, arthrosis and arthralgia.</td>
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<tr>
<td>Aromasin</td>
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<td>Femara</td>
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<td>Type II aromatase inhibitors selectively inhibit aromatase P450 enzyme, reducing the levels of estrogen, with higher target specificity and reduced non-specific side effects.</td>
<td>Risk for developing osteoporosis and joint disorders, i.e. arthritis, arthrosis and arthralgia.</td>
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<tr>
<td>Arimidex</td>
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</tbody>
</table>
## Breast cancer treatments, dates of discovery, uses and common side effects.

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</tr>
</thead>
<tbody>
<tr>
<td>Oncovin, Vincasar, PES, Vincrex (vincristine) and Velban (vinblastine)</td>
<td></td>
<td>R.L. Nobbel and C.T. Beer</td>
<td>Vinca alkaloid</td>
<td>Vincristine is used for the treatment is acute childhood leukemia and advanced-stage breast cancer. Vinblastin is used for the treatment of lymphoma, i.e. Hodkin's disease, advanced testicular cancer, and for advanced breast cancer. Vinca alakaloids were originally isolated from the plant vinca rosea, now named Catharanthus roseus, and commonly known as the Madagascar Periwinkle. They work by serving as mitotic spindle poison, since they bind to tubulin and prevent cell division.</td>
<td>Peripheral neuropathy (numbness of fingers and toes), hyponatremia (lower serum sodium concentrations than normal), constipation, and hair loss</td>
</tr>
<tr>
<td>Nevelbine</td>
<td></td>
<td></td>
<td></td>
<td>Low blood counts, nausea or vomiting, muscle weakness, constipation, pain at injection site, peripheral neuropathy (numbness of fingers and toes), diarrhea, hair loss, and low platelet counts.</td>
<td></td>
</tr>
</tbody>
</table>

*Notes to the year of the drugs' discovery to the year of FDA approval. The discovery to FDA approval period was provided when available. The information was obtained from Li, J.J. (2006). *Laughing Gas, Viagra, and Lipitor: The Human Stories Behind the Drugs We Use*. and from sideeffects.embl.de (2010; Li, 2006)*
References


CHAPTER 2

EFFECTS OF A HYDRO-ALCOHOLIC EXTRACT OF THE TIBETAN ROOT RHODIOLA CRENULATA ON AN ENGINEERED INVASIVE MAMMARY EPITHELIAL CELL LINE

*Rhodiola crenulata*: Taxonomy and History

The root of the Tibetan plant *Rhodiola crenulata* is part of eastern traditional medicine, *R. crenulata* belongs to the family Crassulaceae (orpine family), genus *Rhodiola*. There are at least 200 documented species and subspecies of *Rhodiola*, of which about 20 are used in traditional Chinese medicine. Reports of its use in Tibetan herbal medicine date back to 760 AD. *Rhodiola* plants are usually found growing at high altitudes in barren soils and it is believed that the plant’s adaptation to grow under these conditions contributes to some of the protective and medicinal activities of the genus. Members of this genus are considered “adaptogens”, as they exert a variety of health promoting effects. Rhodiola plants have been previously reported to confer resistance to diabetes, hypertension, fatigue and hypoxia, decrease depression, enhance athletic performance and immunity, and aid in the adjustment to high altitudes (Kelly 2001). The *Rhodiola rosea* species has been shown to have anti-cancer effects in both animals and humans (Udintsev and Shakhov, 1991; Majewska et al., 2006; Kormosh et al., 2006; Skopinska-Rozewska et al., 2008; Skopinska-Rozewska et al., 2008; Zhang et al., 2007). Previous studies determined that *Rhodiola* extracts enhance the efficiency of intracellular DNA repair mechanisms, display antibacterial properties and Tü et al., (2008) have reported that *R. crenulata* displays anti-breast cancer properties.

*Rhodiola crenulata* as a potential therapeutic for cancer

As I have discussed, there is a great need for novel therapeutics to treat breast cancer. Early detection of the disease and an aggressive treatment regime are essential for a good prognosis. Thus far it has been possible to treat cancers with a defined set of molecular markers, such as estrogen receptor and HER-2 on the extracellular cell surface, that would normally transmit potent mitosis-inducing signals and
induce tumor growth. However, it is imperative that treatments for cancers which do not possess such markers be developed. It is of utmost importance that the compound be effective, specific and safe for the patient. Preclinical trials have identified compounds that might be useful to protect against and treat breast cancer. More than half of the approved anticancer drugs currently in have been derived directly or indirectly derived from natural products. In fact, from 1981-2010 close to 80% of all anti-cancer drugs developed originated from natural products. Such “natural” compounds are many times derived from plants (phytochemicals) and have a long history of use in traditional medicine. With this in mind I focused on determining if an extract of an exotic plant, *Rhodiola crenulata* has an anticancer effect against aggressive (highly invasive or metastasizing) cancer cells, which are the main cause of metastasis and cancer recurrence.

Previous studies showed that the hydro-alcoholic extract of *Rhodiola rosea* prevented the growth of solid sarcomas implanted in rats and also decreased metastasis to the liver of cells from implanted metastasizing tumors in the same animal models (Kelly, 2000). In humans, a *Rhodiola sp.* extract reduced the average frequency of relapse in patients with superficial bladder sarcoma. Our lab found that when mice were treated with *R. crenulata* extract and injected with tumor cells, a primary tumor failed to form in 30% of the animals. Furthermore, *R. crenulata* fed to mice injected with breast cancer cells increases the survival time of tumor graft-bearing mice (Tu et al., 2008). Tu et al., found that treatment with the *R. crenulata extract* renders both mouse and human breast cancer cells more susceptible to cell death and it significantly inhibits the migratory and invasive behaviors exhibited by metastatic breast cancer cells.
Breast cancers progress through stages of increasing malignancy by genetic and epigenetic alterations that promote their growth, invasiveness and metastasis. Most times these alterations occur in one or more components of the 18 signature pathways identified by Gráatza, et al. (2011). One such pathway, the Wnt signaling pathway, is deregulated in breast cancer tissues. Aberrant activation of the β-catenin pathway, also known as the canonical Wnt/β-catenin pathway, contributes to the genesis of a wide range of human cancers by giving rise to malignant behavior. This pathway has regulators that keep the pathway in check, and deregulation of such molecules can result in cancer. Secreted frizzled-related proteins (SFRPs) are a family of proteins that antagonize Wnt signaling. It has been shown that SFRP1 is reduced or lost in mammary cancer cell lines as well as human breast tumor specimens (Kloppocki et al., 2004; Lo, et al, 2006; Wong et al., 2002; Zhou et al., 1998). In the absence of SFRPs Wnt protein binds to the Frizzled receptor at a cell’s surface. Wnt binding leads to inhibition of glycogen synthase kinase 3β (GSK3β). GSK3β is an inhibitor of β-catenin, a coactivator of the TCF/LEF family or transcription factors. Upon GSK3β inhibition, β-Catenin is no longer ubiquitinated and targeted for proteosome-mediated degradation, resulting in accumulation of intracellular β-catenin, β-catenin then translocates to the nucleus where it activates the transcription of various genes, amongst them genes involved in the epithelial to mesenchymal transition (EMT) (Polyak & Weinberg, 2009). Our research group recently reported that knock down of SFRP1 in a non-malignant epithelial cell line, 76N TERT (TERT-siSFRP1 cells), results in cells that acquire tumorigenic properties characteristic of aggressive breast cancer cells. These characteristics include resistance to anoikis (anchorage-independent cell death),
up-regulation of CD44 and down-regulation of CD24 cell surface markers and increased invasive behaviors (Gauger et al., 2009).

It has been recently accepted that mammary stem cells reside in adult breast tissue and give rise to progenitor cells. These stem-like cells generate differentiated progeny that populate the breast and form the mammary glands' diverse cell types (Russo et al., 2006). The presence of stem cells in the mouse mammary gland has been clearly demonstrated by several researchers who show that a single stem cell can repopulate a cleared fat pad (Shackleton et al., 2006; Stingl et al., 2006; Liao et al., 2007). It has been shown that mammary stem cells are enriched with a population of cells that can grow under anchorage-independent (AIG) conditions. When in non-adherent culture conditions these cells are capable of surviving, proliferating and forming discrete rosettes of cells that have been termed “mammospheres”. The rosette phenotype is similar to that of embryonic stem cell colonies. Disaggregation and subsequent plating of the mammosphere cells results in the formation of secondary and tertiary mammospheres, indicating the maintenance and the renewal of the stem-like cells. In contrast, non-stem-like cells lose their ability to form spheres by the third round of disaggregation and plating. The mammospheres are enriched with progenitor cells whose plasticity allows them to differentiate into multiple mammary epithelial cell lineages (Dontu et al., 2003). Recently it was discovered that mammary carcinomas also possess a stem-like population of cells. These cells can form “tumorspheres” when placed in non-adherent conditions, very few of these cells are required to form tumors, and they display both similar behaviors and cell surface markers found in normal stem cells (Cariati et al., 2008; Ponti et al., 2005). Al-Hajj et al., (2003) found that as few as 100 carcinoma cells possessing a distinct stem cell surface marker expression pattern of CD44<sup>high</sup>/CD24<sup>low</sup> can form mammary tumors in otherwise healthy mice (Al-Hajj et al., 2003). These observations have led researchers to hypothesize that tumors arise due to either (a) mutations that impart cancer properties to a stem or progenitor cell, or (b) through the de-differentiation of a mutated epithelial cell into a stem/progenitor-like cell (Tan, et al. 2006).
To study the effect of *R. crenulata* on stem-like cells it was essential to include a characterized control cell line that would allow us to correct for confounding variables. We therefore chose to use 76NTert-siSFRP1 cells as our comparative control and human cancer invasive MDA-MB-231 cells. Both cell types exhibit the stem cell-like properties previously described (Gauger et al., 2009; Sheridan et al., 2006), including a mesenchymal-like phenotype, invasive behavior and resistance to apoptosis triggered by anchorage independent conditions (anoikis). The MDA-MB-231 cell line is a classic example of the triple negative breast cancer cell type and it has been used in many studies. The 76NTert-siSFRP1 cell line was developed and described by Gauger, et al., (2009). The 76NTert cells were originally isolated from tissue obtained from a reduction mammoplasty procedure, and were subsequently immortalized by ectopic expression of active subunit of telomerase, hTERT (Kim et al., 1994; Counter et al., 1998; Fan et al., 2005). These cells behave like unaltered human mammary epithelium, i.e., they respond to cell-cell contact growth inhibition. Knocking down SFRP1, a suppressor Wnt/β-Catenin signaling pathway resulted in cells with a malignant phenotype (increased stem cell markers) which are invasive and resistant to anoikis. The work described hereafter was undertaken to determine if *R. crenulata* is capable of attenuating some of the malignant characteristics associated of human breast cancer cells that possess a stem-like phenotype.

Our data demonstrate that treatment of 76NTert-siSFRP1 cells with an extract of *R. crenulata* inhibited their migration and invasion, as compared to untreated cells. Furthermore, *R. crenulata* sensitized the cells to anoikis but did not increase γ-radiation-induced cell death. We provide evidence that the loss of invasive capabilities induced by *R. crenulata* does not occur through the inhibition of an epithelial-to-mesenchymal transition (EMT). Additionally, we clearly demonstrate that *R. crenulata* blocks prevents tumorsphere formation in TERTsiSFRP1 and MDA-MB-231 cells, it alters the expression of the Id family of genes, which are known to regulate the processes of migration, invasion, cell “stemness” and differentiation and it increases the production of reactive oxygen species (ROS), a process recently shown important to inhibit cell migration (McAllister, et al., 2011).
Materials & Methods

Cell Culture

TERT-siSFRP1 cells were routinely cultivated at 37°C in 5% CO₂ and maintained in DMEM/F12 (GIBCO, Grand Island, NY) supplemented with the following components from GIBCO: 1% FBS, 1X Antibiotic-Antimycotic (100X), and 20 µg/mL Gentamycin, and 2µg/mL puromycin. The following components from Sigma (St Louis, MO) were also used as medium supplements; 50 µM L(+) Ascorbic acid sodium salt, 1ng/ml Cholera Toxin Vibrio, 12.5 ng/ml Epidermal Growth Factor (Murine Submaxillary Gland, 324856, Calbiochem), 2 nM β-Estradiol (E-2257, Sigma), 0.1 mM Ethanolamine (E-9508, Sigma), 1 µg/ml Hydrocortisone-Water Soluble (H0396, Sigma), 1 µg/ml Human Insulin solution (I-9278Sigma), 0.1 mM O-Phosphorylethanolamine (P-0503, Sigma), 35 µg/ml bovine pituitary extract (P-1476, Sigma), 15 nM Sodium Selenite (S-1382, Sigma), 10 µg/ml freshly made Human apo-Transferrin (T-2252, Sigma), and 10 nM 3,3',5-Triiodo-L-thyronine Sodium salt (T-5516, Sigma).

Fluorescence Activated Cell Sorting (FACS)

76NTERT-siSFRP1 cells were grown to 70% confluence and pre-treated with either 0.1% EtOH or 100 µg/ml R. crenulata (final concentrations). Sterile 30 mm cell culture dishes were coated with 2 ml of 1% agarose/DMEM, which was allowed to polymerize. The polymerized 1% agarose/DMEM created a barrier that prevented cellular attachment to the surface of the culture dish. After 24 hours, the pre-treated cells were trypsinized and centrifuged at 1,000 rpm. The cell pellet was re-suspended in 6 ml of growth medium, and 2 ml of cell suspension were added to each of the 1% agarose/DMEM coated dishes. Following a 24 hour incubation, the media and
cells were collected. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes and the supernatant was carefully removed via aspiration. The cell pellet was carefully re-suspended in ice-cold 1X PBS, pH7.2, transferred into a round bottom 12x75 mm plastic culture tube (VWR international, NJ, USA), and incubated with 1\(\mu\)g/ml propidium iodide (Invitrogen) on ice and in the dark for 15 minutes to stain dead cells. The ratio of dead cells/total cells was determined by flow cytometry (BD FACSCalibur\textsuperscript{TM}, BD Bioscience, San Jose, CA).

**Migration and Invasion Assay**

76NTERT-siSFRPI cells were grown to 70% confluence and pre-treated with either 0.1% Ethanol (EtOH) or 100 \(\mu\)g/ml \textit{R. crenulata} (final concentrations). After 24 hours, pre-treated plates were trypsinized. Media and cells were collected, centrifuged at 1,000 rpm, and the pellet was re-suspended to a final concentration of 1 x 10\(^6\) cells/ml in serum-free media. A total of 5 x 10\(^5\) cells/well were seeded in serum free media in either BD Biotrak control chambers or Matrigel\textsuperscript{TM} invasion Boyden chambers (BD Biosciences). Media containing 10% FBS was used as chemoattractant in the lower compartment of the Boyden chamber. Following a 22 hour incubation, the assay chambers were removed and placed for 10 min in 10% formalin to fix the cells which were then stained for 10 min with 10% Crystal Violet, and rinsed 3X with dH2O. Non-migrating/invading cells were removed from the upper surface of the membrane by scrubbing the insert with a cotton tipped swab that had been previously moistened with 1X PBS, pH7.2. The insert was carefully removed from the chamber with a scalpel, placed on a microscope slide (cell side up) and mounted with Richard-Allan Scientific Cytoseal\textsuperscript{TM} 60 (Thermo Scientific, Portsmouth, NH). Images were captured at a 10X magnification with an Olympus BX41 light microscope using SPOT Basic Imaging Software (SPOT Imaging...
Solutions, a division of Diagnostic Instruments, Inc, Sterling Heights, MI). Images were taken of three representative areas of each sample slide. The stained cells that crossed to the underside of the membrane were counted and the results were analyzed.

**RNA Isolation and Microarray Analysis**

76NTERT-siSFRP1 cells were treated with 10% EtOH or 100 μg/ml *R. crenulata* and after 24 hours, RNA was isolated using a QIAGEN Quickspin kit according to the manufacturer’s instructions. Agilent 4X44K human microarrays were performed by the Genomics Core Facility at University of North Carolina at Chapel Hill according to the manufacturers Amplification protocol. Four RNA harvests at 60-80% confluence of the vehicle treated 76NTERT-siSFRP1 cells were pooled and labeled with Cy3, while separate harvests (n=4) of the *R. crenulata* treated cells were labeled in Cy5. Agilent feature extraction, loess normalization, and filtering of all spots with signal <10 dpi was performed prior to supervised analysis. One class Significance Analysis of Microarrays (Tusher, Tibshirani et al. 2001) was used to identify genes that were significantly altered in *R. crenulata* treated cells relative to vehicle treated cells with a FDR <5%.

RNA samples for QRT-PCR were prepared as follows 76NTert-siSFRP1 cells were treated with 10% EtOH or 100 μg/ml *R. crenulata*. Following a 24 hour incubation total RNA was isolated with TRIzol® Reagent (15596-018, Ambion by Life Technologies Corporation, Molecular Research Center, Inc., Molecular Research Center, Inc., Carlsbad, CA) following the manufacturer’s protocol. Samples were re-suspended in UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen Corporation, Carlsbad, CA). The quantity and quality of the RNA in each sample was determined by spectrophotometric analysis using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).
**Statistical Analysis**

Results were analyzed using a student’s t-test or a two-factor analysis of variance (ANOVA). 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 (Prizm, GraphPad Software, Inc, San Diego, CA).

**Results**

Cellular metastasis takes place when epithelial cells in a primary tumor leave their site of origin, digest and break through the extracellular matrix, migrate into blood vessels and invade secondary sites. It has been previously shown that 76NTERT-siSFRP1 cells are an extremely migratory and invasive mammary epithelial cell line (Gauger et al., 2009). Since *R. crenulata* inhibits the migration and invasion of MDA-MB-231 breast cancer cells (Tu et al., 2008), a cell line previously shown to lack SFRP1 expression due to hypermethylation of the SFRP1 promoter (Lo, et al, 2006), we hypothesized that the motile and invasive behavior observed in 76NTERT-siSFRP1 cells would be abolished if they were grown in the presence of *R. crenulata*. Considering that metastasis is likely to occur when cells become more migratory, we decided to test the effects of *R. crenulata* on 76NTERT-siSFRP1 cells utilizing a simple scratch wound assay (*Figure 1a*). The images that we obtained illustrated that after just 3 h, 76NTERT-siSFRP1 cells treated with 0.1% EtOH (vehicle) began to migrate and fill in the wound whereas treatment of cells with 100 µg/ml *R. crenulata* prevented the motility of these cells. After 6 h, *R. crenulata* continued to block migration. At 24 h, 76NTERT-siSFRP1 cells completely filled in the scratch wound whereas cells were grown in the presence of *R. crenulata* did not; at 24 h wound closure had just begun. Next, cells were plated in BD BioCoat control chambers or Matrigel Invasion Chambers (BD Biosciences). The cells capable of migrating through the 8 µm pore towards a chemo-attractant were stained with 10% crystal violet (*Figure 1b*). Quantification of the stained cells revealed that 76NTERT-siSFRP1 cells treated with *R. crenulata* failed to migrate through the membrane pores, indicating that
they were significantly less migratory than vehicle treated cells (Figure 1c). Cells that were able to invade the reconstituted basement membrane (Matrigel) through an 8 μm pore towards a chemo-attractant were stained with 10% crystal violet, Figure 2B clearly illustrates and Figure 1C quantitatively confirms that *R. crenulata* blocked the invasiveness of 76NTERT-siFRP1 cells.

Epithelial to mesenchymal transitions (EMT) are implicated in the conversion of early stage tumors into invasive malignancies. EMT is a process where epithelial cells lose polarity, lose cell-cell contacts and hence cell-cell contact inhibition, and undergo a dramatic remodeling of their cytoskeletons, resulting in cells with a mesenchymal morphology. Interestingly, Mani et al., 2008, showed that mammary epithelial cells forced to undergo EMT acquired stem cell-like characteristics (Mani et al., 2008). Both 76NTERT-siFRP1 and MDA-MB-231 cell lines possess a fibroblast-like morphology and express several genes associated with EMT in a pattern that is consistent with the pathophysiological phenotype of EMT (Gautger et al., 2009; Olmeda et al., 2007; Mbalaviele et al., 1996; Bindels et al., 2006; Eger et al., 2005). Since the data described thus far demonstrates that *R. crenulata* impedes several behaviors associated with invasion, we wanted to establish whether the genes implicated in EMT were affected by *R. crenulata* treatment. Real-time PCR analysis of well characterized EMT associated genes revealed that the mRNA expression of these genes did not differ between vehicle and *R. crenulata* treated cells (Figure 2).

The pathogenesis of breast cancer involves the aberrant expression and altered function of the genes involved in cell-cycle regulation and cell death. Tu et al., previously demonstrated that *R. crenulata* induces cell death in both human and murine breast cancer cell lines in the absence of serum (Tu et al., 2008). Therefore, we sought to determine whether *R. crenulata* would cause cell death in 76NTERT-siFRP1 cells. We found that *R. crenulata* treated 76NTERT-siFRP1 cells underwent significantly more death both in the presence and absence of ionizing radiation (IR), although IR treatment did not further enhance *R. crenulata* induced cell death (Figure 3a). Normal non-malignant adherent cells are strongly dependent on adhesion to the extracellular matrix for cell proliferation and undergo cell death if they are detached from the substratum, in a process known as anoikis (Frisch and Francis, 1994).
Resistance to anoikis has been suggested as a prerequisite for cancer cells to metastasize. In contrast, tumor cells can survive and grow without adhesion to a substratum. With this in mind we next tested the ability of *R. crenulata* to sensitize the anoikis-resistant 76NTERT-siSFRP1 cells to anchorage-independent cell death. Twenty-four hours after seeding the cells, FACs analysis of Propidium Iodide incorporation was performed to compare events of cell death between vehicle and *R. crenulata* treated cells. In cells grown unattached, more cell death occurred when cells were treated with *R. crenulata* as compared to the vehicle treated cells (*Figure 3b*). Moreover, after 48 h of *R. crenulata* treatment, 76NTERT-siSFRP1 cells remained susceptible to anchorage-independent induced cell death (*Figure 3b*).

Next, we sought to determine whether an established invasive breast cancer cell line, MDA-MB-231 cells, would be sensitized to anoikis when grown in the presence of *R. crenulata*. We clearly show that, in anchorage-independent growth conditions at 24 and 48 h, significantly more *R. crenulata* treated MDA-MB-231 cells died as compared to vehicle treated control cells (*Figure 3b*).

Both non-malignant and malignant mammary epithelial cells with stem-cell characteristics, such as a CD44$_{\text{high}}$/CD24$_{\text{low}}$ cell surface marker pattern, are capable of forming mammosphere/tumorspheres in suspension culture (Ponti et al., 2005). It has previously been demonstrated that both 76NTERT-siSFRP1 cells and MDAMB-231 cells exhibit the CD44$_{\text{high}}$/CD24$_{\text{low}}$ phenotype and form tumorspheres when cultured under the appropriate conditions (Gauger et al., 2009; Sheridan et al., 2006).

We wanted to determine whether *R. crenulata* affects the CD44$_{\text{high}}$/CD24$_{\text{low}}$ surface receptor expression exhibited by these cell lines and found that there was no difference between vehicle and *R. crenulata* treated cells (Gauger, et al., 2008). Next, to establish whether *R. crenulata* is able to abolish mammosphere formation, we seeded 76NTERT-siSFRP1 and MDA-MB-231 cells at a single cell density under conditions that would allow for tumorsphere formation and treated with vehicle or *R. crenulata* for 10 days. *Figure 4a* illustrates that when cells were treated with *R. crenulata*, the cells were unable to form tumorspheres (Gauger, et al., 2008). We then quantified the extent to which *R. crenulata* impeded the ability of dissociated 76NTERT-siSFRP1 and MDA-MB-231 tumorsphere cells from forming secondary
and tertiary tumourspheres. Our data clearly indicate that there was a significant reduction in tumoursphere development (Figure 4b).

To gain a better understanding of the cellular process affected by *R. crenulata*, other than EMT that could explain the anti-invasion phenotype, we performed microarray analysis of mRNA transcripts from 76NTert-siSFRP1 cells treated with or without *R. crenulata*. I performed a microarray analysis of the microarray data with the Ariadne 6 software and identified signaling pathway with significant changes in the pattern of expression of its components. Of interest I identified the ID1, ID2, ID3 pathway (Figure 5a). Our microarray data indicated that, compared to cells treated with vehicle, *R. crenulata* treated cells displayed upregulated transcription of ID2 and downregulation of ID1 and ID3 transcription. The Id (Inhibitor of differentiation or inhibitor of DNA-binding) proteins are basic Helix-Loop-Helix (bHLH) proteins that lack a DNA-binding region and act as dominant negative inhibitors of other bHLH transcription factors that regulate differentiation-specific gene expression. The Id proteins play several critical roles in both normal and transformed tissues, including the mammary gland (Desprez et al., 2003). These proteins are able to promote cell proliferation and survival by activating growth promoting and blocking tumour suppressive pathways (Yokota and Mori, 2002; Ling et al., 2003; Swarbrick et al., 2005; Ling et al., 2002). Ids have also been implicated in stem cell renewal and maintenance and are often down-regulated during stem cell differentiation (Jung et al., 2009; Nogueira et al., 2000; Perry et al., 2007). It has been previously shown that Id-1 is frequently up-regulated in many types of human cancer and that its expression levels are also associated with an advanced tumour stage (Wong et al., 2004). In addition, a close association between Id-1 expression and invasion has been demonstrated in both non-cancerous and cancer cells (Desprez et al., 1998; Takai et al., 2001; Lin et al., 2000). Since the mechanism by which *R. crenulata* blocks invasion, sensitizes cells to anoikis, and inhibits tumoursphere development did not involve EMT repression, we decided to investigate whether *R. crenulata* may be affecting the aforementioned processes by altering the expression of Id genes. Using real-time PCR analysis we sought to confirm the validity of the data obtained via microarray analysis. We confirmed that 24 and 48 h after *R. crenulata* treatment, in both 76NTERT-siSFRP1 and MDA-MB-231 cells, Id-1 and
ld-3 mRNA levels were remarkably diminished when compared with vehicle treated cells. In contrast with ld-1 and ld-3, R. crenulata significantly increased the expression of mRNA of ld-2, a pro-differentiation factor, in both 76NTERTsiSFRP1 and MDA-MB-231 cells (Figure 5b).

Discussion

In this study, it was demonstrated that several important cellular processes of triple negative breast cancers are drastically inhibited by an extract of R. crenulata. While the cells treated with R. crenulata did not display a change in cell morphology, the hydro-alcoholic extract of this medicinal plant inhibits migration, invasion, resistance to anoikis, and the capability of tumor sphere formation. We propose that one mechanism by which R. crenulata exerts such drastic effects on these particular actions is by regulating ROS production and the transcription of ld genes, a family proteins known to play a role in signaling pathways involved in differentiation, cell cycle, stem cell maintenance and tumorigenesis. Although further studies are required to define the pathways affected by the R. crenulata, the extract and compounds of R. crenulata may prove to be excellent candidates for the treatment of aggressive breast tumors and the prevention of metastases.

The finding that both lds were similarly regulated by R. crenulata was not surprising considering that ld-1 and ld-3 have extensive sequence homology and similar patterns of expression during embryogenesis and in mouse adult tissues (Ellmeier and Weith, 1995). Interestingly, Shuno et al, recently showed that when ld-1 and ld-3 were stably knocked down in human pancreatic cells, proliferation and migration are significantly reduced in vitro and peritoneal metastases are significantly blocked in vivo (Shuno et al., 2008). The down-regulation in mRNA transcript levels of ld1 and ld3 after treatments with R. crenulata that were observed agree with the process of differentiation as reported by Lyden and colleagues (1999). Their group found that embryos with a double ld1 and ld3 knockdown (ld1-/- ld3-/-) are not viable, one cause being the premature differentiation of cells in neuronal tissue, specifically premature differentiation of the neuronal lineage. Additionally, Lyden, et al, found that adult ld1+/ ld3-/- mice with tumor xenografts present tumors with inhibited growth, as compared to their wild-type counterparts and such
tumors were also necrotic and hemorrhagic due to angiogenic defects not found in their wild type counterparts. In another report Tsuchiya, et al., (2005) reported that gastric cancer cells (cell line MKN45) with an Id1, Id3 double knockdown displayed decreased proliferation, decreased migratory capabilities and failed to establish peritoneal metastatic nodules. Therefore, it is possible that the *R. crenulata* induced decrease in Id1 and Id3 triggers a process of differentiation in cancer cells with stem cell-like traits that attenuates malignant and stem cell characteristics of 76NTERT-siSFRP1 and MDA-MB-231 cells by reducing the expression of Id-1 and Id-3. A pro-differentiation effect seems possible if we consider that *R. crenulata* treatment of both cell types resulted in an increase of Id-2 expression. Interestingly, unlike Id-1 and Id-3, the expression of Id-2 is up-regulated *in vitro* and *in vivo* as mammary epithelial cells lose their ability to proliferate and begin the process of differentiation (Parrinello et al., 2001). Moreover, Itahana et al., (2003) demonstrated that Id-2 is highly expressed in well differentiated human breast cancer cells as compared to very aggressive metastatic cells. Also, in comparison to *in situ* carcinomas, Id-2 protein expression is markedly reduced in human biopsies from aggressive and invasive carcinomas. These researchers demonstrated that reintroduction of Id-2 into metastatic breast cancer cells, blocks their invasive phenotype (Itahana et al., 2003). More recently McAllister and colleagues (2011) demonstrated that treatment of malignant breast cancer cells with cannabinoid (CBD) resulted in activation of the ERK signaling pathway, down-regulation of Id-1 to almost undetectable levels, and a significant up-regulation of Id-2. CBD treatment also inhibited the capability of mouse mammary cancer cells to proliferate and to invade other tissues and it. Taken together, previous evidence and our current results suggest that *R. crenulata*, inhibits cellular migration and invasion in a mechanism that possibly involve the down-regulation of Id-1 and Id-3 and the up-regulation of Id-2 expression.
facilitates the migratory and invasive properties of 76NTert-siSFRP1 cells. *R. crenulata* blocks the cellular migration and invasion of 76NTert-siSFRP1 cells. (A) TERT-siSFRP1 cells were plated in 30 mm dishes and allowed to reach 100% confluence and treated with the vehicle tip was utilized to generate a wound (scratch) down the center of the plate. The schematic of this experiment is shown. Images were captured at 24 hours; scale bar 100 μm. (B) TERT-siSFRP1 cells treated with vehicle or *R. crenulata* were plated in BD BioCoat control chambers. Cells capable of migrating through the 8 μm pore or invading the reconstituted basement membrane were towards a chemoattractant were stained with 10% crystal violet and counted. Images were captured at 10X magnification; scale bar 25 μm. (C) Experiments were repeated three times and the numbers of cells within a representative 10X field from each image bar represents SEM of the cell number. *p<0.05, ***p<0.001 (significantly different from vehicle treated cells taken from (Gauger, Hugh, Troester, & Schneider, 2009))
Figure 6. The anti-invasive effects of the R. communis leaf extract (EOH) or R. communis root extract (100 μg/mL) on HBE cells were incubated with 0.1% ethanol for 24 h. After treatment, total RNA was isolated from the cells using TRIzol according to the manufacturer's QIAGEN protocol. QRT-PCR analysis was performed on the samples. β-Actin was used as internal control.
Figure 7. *R. crenulata* extract sensitizes 76NTert-siSFRP1 cells to AIG induced death. 76NTert-siSFRP1 cells were treated with either 1:00 Vehicle (10% EtOH) or *R. crenulata* extract (100μg/mL) and incubated for 24h. Cells were trypsinized and plated under anchorage-independent growth conditions (on an agar coated plate). Cells were kept under continued treatments. PI incorporation was determined via FACS analysis.
Figure 8. *R. crenulata* blocks tumorsphere formation. A total of 500 TERT-siSFRP1 and 500 MDA-MB-231 cells were plated in low-attachment 96 well plates, treated with vehicle or *R. crenulata*, and cultured for 10 days. (A) Phase contrast images were captured at days 1, 5, 7 and 10. Images were captured at 20X magnification; (B) *In vitro* quantification of tumorspheres formed by cells as described. Bars represent SEM of the number of spheres formed/500 seeded cells. *p* < 0.05 (significantly different from vehicle treated cells using student’s t-test). (Gauger et al., 2009)
Figure 9. *R. crenulata* extract inhibits the mRNA expression of Id1 and Id3. 76N-Tert-siSFRP1 cells at ~90% confluence were incubated with vehicle (0.1% EtOH) or *R. crenulata* extract (100μg/mL) for 24h. After treatment, total RNA was isolated from the cells with TRIzol, according to the manufacturer. Microarray chip hybridization and initial statistical analysis was performed by collaborators at the Troester laboratory. (A) Microarray data analysis using the Ariadne P6 software revealed changes in expression levels of members of the Id family of proteins. (B) QRT-PCR analysis was performed to verify and compare Id-1, Id-2, and Id-3 mRNA expression levels in 76N-Tert-siSFRP1 cells treated with vehicle or *R. crenulata*. β-Actin and RAP1A were used as normalizers.
References


Lyden, D., Young, a Z., Zagzag, D., Yan, W., Gerald, W., O’Reilly, R., Bader, B. L., et al. (1999a). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature, 401*(6754), 670–7. doi:10.1038/44334
Lyden, D., Young, a Z., Zagzag, D., Yan, W., Gerald, W., O’Reilly, R., Bader, B. L., et al. (1999b). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature, 401*(6754), 670–7. doi:10.1038/44334


doi:10.1038/sj.onc.1208188

Takai, N., Miyazaki, T., Fujisawa, K., Nasu, K., & Miyakawa, I. (2001). Id1 expression is
associated with histological grade and invasive behavior in endometrial carcinoma. *Cancer
letters, 165*(2), 185–193.


Tsuchiya, T., Okaji, Y., & Tsuno, N. (2005a). Targeting Id1 and Id3 inhibits peritoneal

Tsuchiya, T., Okaji, Y., & Tsuno, N. (2005b). Targeting Id1 and Id3 inhibits peritoneal

Tu, Y., Roberts, L., Shetty, K., & Schneider, S. S. (2008). Rhodiola crenulata induces death and
doi:10.1089/jmf.2007.0736

Udintsev, S., & Shakhov, V. (1991). The role of humoral factors of regenerating liver in the
development of experimental tumors and the effect of Rhodiola rosea extract on this

related protein and Wnt-signaling molecules in invasive human breast tumours. *The Journal
of Pathology, 196*(2), 145–153. doi:10.1002/path.1035

289.

physiology, 190*(1), 21–8. doi:10.1002/jcp.10042

salidroside on hydrogen peroxide-induced apoptosis in SH-SY5Y human neuroblastoma
doi:10.1016/j.ejphar.2007.01.089

frizzled homolog in apoptosis and its down-regulation in breast tumors. *International
al.)1097-0215(19980925)78:1<95::AID-IJC15>3.0.CO;2-4
CHAPTER 3

EFFECT OF R. CRENULATA ON CELL POLARITY AND CYTOSKELETAL REMODELING

From Migration to Invasion: How Cancer Cells Break Free, Where They Go and Why

Defining metastasis

The process of metastasis is the primary cause of recurrence of breast and other cancers. Our current knowledge of the process of metastasis tells us that there are many molecular, biochemical and cellular processes involved in the process of cancer cell metastasis. Metastasis can be viewed as an epithelial cancer cell’s successful acquisition of the migratory machinery. However, the process of migration is in itself complex and tightly regulated. The growth of normal epithelia is controlled in part by cell-cell contact growth inhibition. Two premises govern cell contact inhibition (Heckman (2009). The first premise is that cultured cells migrate on the surface of the provided substratum but they do not adhere to the upper surface of another cell in the same non-confluent culture, nor do they use other cells as substratum. The second premise is that two cells that make contact at their leading edge (considered the front of the cells or the side towards which the cell is moving), where ruffling takes place, cease motility and the ruffling at the edge where the two cells make contact. The sides where the cells do not make contact maintain ruffles and the cells can change direction and move towards the ruffling (Heckman (2009). Cell contact inhibition thus ensures that a cell monolayer is formed in culture and
a defined number of epithelial layers in tissues. In the event of an injury, such as a skin wound, epithelial cells migrate towards the free space until they contact another cell, at which point they stop. If the damage is great, inflammatory cues, i.e. cytokines and growth factors, promote the migration of cells to a site of injury and subsequent repair of the tissue. Cancer cells do not display this behavior. When presented with free space cancer cells will migrate to the free space but fail to cease motility and ruffling once a cell-cell contact is made. Additionally, transformed cells will drive their leading edge under or over another cell rather than stopping their progression (Heckman (2009). It is therefore reasonable to state that cancer cells display no contact inhibition of locomotion.

**How cancer cells orchestrate their “escape”**

Cell migration can be subdivided into five general processes: (1) cell polarization, (2) protrusion formation at the leading edge, (3) adhesion formation, (4) translocation, and (5) retraction of the trailing edge. During polarization an individual cell responds to a migration-inducing signal and accumulates the components necessary for the formation of protrusions in the leading edge, the side or edge of the cell that is free of contact. During the second step, polymerization of actin at the leading edge extends the cell membrane, forming a protrusion directed towards the migration signal. This process is usually coordinated by GTPase of the Rho family mostly Cdc42 and Rac, and the protrusions formed are varied in morphology and function (Olson and Sahai (2009). There are three main types of protrusions: lamellipodia (a broad zone of F-actin containing, spread membrane at the leading edge of the cell), filopodia (numerous, thin F-actin rich, needle-like protrusions at the leading edge), and pseudopodia (an elongated
protrusion with a prominent “ruffled” zone of F-actin-spread membrane at the leading edge) (Olson and Sahai (2009). The third step is adhesion of the protruding edge. During this step new contacts are formed between the cell and its substratum and it usually directed by Rαc. The fourth step, translocation, involves the contraction of the acto-myosin complexes at the trailing edge (the “back” of the cell). Contraction of the actin cytoskeleton by myosin results in a “pulling” motion that moves the rear side of the cell towards the leading edge. Contraction is usually orchestrated by Rho. The fifth step, retraction, involves the dissolution of remaining adhesions at the trailing edge which allows the back end of the cell body to complete the translocation (Ridley, Schwartz et al. (2003); Yamazaki, Kurisu et al. (2005). Although the process of migration can be subdivided into these five processes, it is important to keep in mind that these steps don’t always take place separately, but rather, they occur in an overlapping sequence. When polarized movement occurs in cohesive sheets of cells, the processes are maintained, but the polarization occurs throughout the sheet rather than within individual cells (Ridley, Schwartz et al. (2003). Therefore the cells closest to the free space become the sheet’s leading edge and the once farthest from the free space become the trailing edge. Hence, the coordinated migration processes allows a single cell or a group of cells to migrate with directionality.

Pathologists have always recognized loss of cell polarity of epithelial cells as one of the earliest signs of cancer development (Banks and Humbert 2008; Banks, Pim et al. 2012). This notion has been widely accepted because of observed changes in epithelial cell morphology after an epithelial to mesenchymal transition (EMT) has taken place. Cells that undergo EMT display loss of cell-cell contact and adherence junctions
Figure 10. Polarized epithelium. A normal epithelium consists of a single or multiple layers of cells that line a “hollow region” called a lumen. These cells are polarized, possessing an apical region (lumen side) and a basal side (basal lamina side). A basal lamina lines the epithelial layer and underneath it the extracellular matrix connects the epithelium with the connective tissues. Epithelial cells are connected to each other via structures called junctions. Such junctions stitch the cell membranes together (tight junctions), function as hollow channels that allow cell communication and transfer of molecules (gap junctions), allow cell-to-cell attachment and communication via extracellular and intracellular (cytoskeletal) protein complexes (adherens junctions), and bind cells via keratin fibers that allows the epithelium to resist sheering forces (desmosomes). Upon induction of an EMT epithelial cells lose their cell-cell contacts, lose their epithelial polarity and become motile. Some cells will break through the basement membrane and invade other tissues.

would expect that junction proteins, cytoskeletal components, proteins of the polarity complexes and other polarity-related regulatory genes were delocalized or down-regulated in human tumors and metastatic cells. Nevertheless these components are present in metastatic cells, but the role of the cell polarity machinery is repurposed (Banks and Humbert (2008); Banks, Pim et al. (2012)). I therefore focused the next aspect of my investigation into the effect of *R. crenulata* on cytoskeletal remodeling, cell polarity and cell migration.
Though the many components of the cell polarity machinery can cause the transformation of a cancer cell into a malignant type there is one set of molecular players that are ultimately required for efficient migration: the actomyosin cytoskeleton. Since the actin cytoskeleton is the ultimate “highjack” target of metastatic cells that allows cell movement, I decided to focus part of my study into the Rho family of cytoskeletal regulating GTPases, which are essential for cell migration.

**Rho GTPases, Migration, and Invasion**

The Rho family of guanosine triphosphate (GTP)-binding proteins (GTPases) are pivotal for the organization of the actin cytoskeleton and adhesion and for the formation of lamellipodia and filopodia (Ridley, Schwartz et al. (2003); Yamazaki, Kurisu et al. (2005). Rho GTPases serve as molecular switches, cycling between active GTP and inactive GDP bound states (Yamazaki, Kurisu et al. (2005). The molecular switch is controlled by Guanine Nucleotide Exchange Factors (GEFs), GTPase Activating Proteins (GAPs) and GDP-Dissociation Inhibitors (GDIs). GEFs catalyze the conversion of GDP giving rise to Rho-GTP (activation), GAPs accelerate the hydrolysis of GTP to GDP (inactivation), and GDIs prevent the dissociation of Rho from either nucleotide and it binds to Rho causing it to cycle between the membrane and cytosol (Schmidt and Hall (2002). This molecular switch system allows the cell to fine tune and control the activity of Rho GTPases and the maintenance of a gradient of active Rho GTPase at distinct regions of a cell. The gradients are necessary for adequate cell polarity and directional migration. The Rho family of proteins regulates the cell cycle, modulates levels of CDKI proteins, is necessary for Rac-induced cell cycle entry, and is implicated in both pro- and anti-apoptotic processes (Sahai and Marshall (2002). RhoA induces the formation of
stress fibers and adhesion foci and is necessary for metastasis of cancer cells (Arthur and Burridge (2001). Wilkinson (2005) and colleagues found that colorectal carcinoma cells treated with blebbistatin, a myosin II inhibitor resulted in what they called a “collapsed” morphology. The group named the observed phenotype collapsed morphology to distinguish the spherical body arising after inhibition of the cells’ contractility machineries from the so called ‘rounded’ or blebbing morphology that is sometimes observed in cells grown on three-dimensional matrices (Wilkinson, Paterson et al. (2005). Inhibition of Rho kinase (ROCK) with Y-27632 also resulted in the collapsed cells with protruding fibers that were F-actin-poor and microtubule-rich. Wilkinson, Paterson et al. (2005) also reported that the collapsed morphology was observed in the highly metastatic, triple negative (estrogen receptor negative, progesterone receptor negative, Her2/neu receptor negative) breast cancer cell line MDA-MB-231 when either the Rho effector ROCK or the Cdc42 effectors were inhibited (Wilkinson, Paterson et al. (2005). Their results suggest that disruption of cell polarity or of GTPase gradients results in cell collapse. Further evidence was presented by (Arthur and Burridge (2001)) who showed that localized RhoA inactivation by p190RhoGTPase-activating protein (190RhoGAP) contributes to cell spreading and migration. When the group transfected cells with a GAP deficient form of p190GAP (namely HA-p190RhoGAP-R1283A), a dominant negative form of p190RhoGAP, the transfected cells displayed increased activity of RhoA. The cells with active RhoA lacked ruffles and possessed robust stress fibers. Additionally, cells with high levels of active RhoA exhibited lower cell spread area, and barely any protrusions in response to a scratch wound in comparison with the mock control. On the other hand, overexpression of wild-type p190RhoGAP, which inhibits RhoA, resulted in
increased number of and longer protrusions than control cells. The group concluded that p190GAP inhibition of RhoA is necessary for cell spreading and migration (Arthur and Burridge (2001)).

In chapter 2 we demonstrated that R. cremulata inhibits the proliferation and the migratory and invasive properties of the engineered 76NTert-siSFRP1 cell line and of MDA-MB-231 triple negative cancer cells in vitro. We also showed that the R. cremulata extract makes these cell types susceptible to death, by a mechanism that we have demonstrated to be independent of reversion of an epithelial-to-mesenchymal transition (EMT). Because MDA-MB-231 cells have a complex genetic background with mutations that have yet not been characterized, we took advantage of a well characterized system of cell migration that occur during the development of frog embryos (Xenopus laevis). Matthews, et al. (2008) have shown that members of Rho family of GTPases regulate X laevis cranial neural crest cell migration both in vitro and in vivo. The group found that Syndecan-4 (Syn-4), a proteoglycan involved in the migration of cells in vitro, inhibits Rac activity, while the planar cell polarity (PCP, a process referring to the polarization of cells in a sheet, or plane (Jones and Chen, 2007)) modulator Disheveled (Dsh) activates RhoA and consequently the RhoA effector ROCK. Additionally, the group found that ROCK inhibited Rac activity (Matthews, et al. 2008). The results of such study suggest that the activities of Syn-4 and PCP come together, polarizing and restricting the formation of cell protrusions to the front of the cell, which allows the cells to migrate with directionality. We therefore sought to determine if R. cremulata affects cell migration during early frog embryogenesis.
In this study we employ \textit{X. laevis} embryos and MDA-MB-231 cells to investigate the mechanisms by which an extract of \textit{R. crenulata} prevents cell migration and metastasis of cancer cells and to define if the extract also affects non-cancer migrating cells. We hypothesize that, in \textit{X. laevis}, \textit{R. crenulata} may inhibit proper cell migration during embryo development by altering the Rho signaling in the migrating tissues, thus disrupting tissue morphogenesis (homeostasis is cell proliferation). I show that \textit{R. crenulata} causes a shift in intracellular ROCK1 distribution in MDA-MB-231 cells. We also showed that migration of CNCs is inhibited by \textit{R. crenulata} and that this process is at least partially reversible by chemical inhibition of ROCK and, finally, I report activity guided fractionation data identifying at least one of the phytochemicals responsible for the anti-migratory action of the hydro-alcoholic \textit{R. crenulata} extract.

**Materials and Methods**

**Cell Culture**

MDA-MB-231 cells were routinely cultivated at 37°C in 5% CO$_2$ and maintained in DMEM (High Glucose) (11995, GIBCO, Grand Island, NY) supplemented with 10% FBS, and 20 µg/mL Gentamicin (15710072, GIBCO). For all assays MDA-MB-231 cells were acclimated low serum containing medium by changing them into DMEM (High Glucose) supplemented with 1% FBS, and 20 µg/mL Gentamicin, and cultivated at 37°C in 5% CO$_2$ for at least half an hour prior to being treated.
Scratch Wound Migration Assay

MDA-MB-231 cells were cultured in 24-well plates, each well containing one culture grade microscope cover glass (12-545-82 12CIR-1D, Fisher Scientific). When cells the cells reached 70% confluence they were acclimated into low serum medium (as described above) and pre-treated with 100μg/mL of R. crenulata extract or 0.1% EtOH. A scratch wound was made on each culture by scraping the cells once with a sterile P-10 pipette tip. The cells were then washed gently with PBS (pH 7.4), being careful to avoid the disruption of the cell monolayer by the stream of PBS. Each well was replenished with fresh medium containing the 100μg/mL R. crenulata or 0.1% EtOH treatment. Cells were incubated at 37°C under 5% CO₂. Following the treatments cells were fixed with 4% paraformaldehyde in 1X PBS, pH 7.2, stained with Phalloidin and mounted with DAPI-containing mounting medium (Vectashield, Vector Labs). Epifluorescence microscopy was performed and analyzed with a Nikon TE2000u microscope and Metavue software.

For live wound healing assay the cells I performed a CellPlayer™ Cell Migration Assay. Briefly, MDA-MB-231 cells were cultivated in 24 or 96-well Essen ImageLock plates (Essen Bio Science, Ann Arbor, Michigan) as described above. Gentle removal of confluent cells was done to generate scratch wounds of reproducible width using the 4 or 96-pin Wound Makers (Essen Bioscience). Subsequently the cells were washed three times with 1XPBS to remove any remaining loose cells. The corresponding treatment medium was added to the cells which were then cultured in an incubator equipped with a High Definition (HD) phase contrast imager (Essen Bio Science). Automated image acquisition was performed every 45 minutes or every hour with the IncuCyte software (Essen Bio Science). Image processing metrics were also performed with IncuCyte
software. Statistical analysis of the mean relative wound density was done using GraphPad Prism4 software.

Fluorescence Immunocytochemistry

MDA-MB-231 cells were seeded in 24-well cells containing culture grade cover-glasses at a density of \(1 \times 10^5\) cells per well. The cells were cultured in their corresponding culture medium. Cells were treated with vehicle or \textit{R. crenulata} extract and incubated at 37°C under 5% CO\(_2\). Following the treatments and incubation the cells were fixed and permeabilized. Immunocytochemistry was performed with a 1:200 dilutions of primary rabbit polyclonal IgG against human RhoA (6739) (Cell Signaling), rabbit polyclonal IgG against ROCK1 (Santa Cruz Biotechnology®; Inc., Santa Cruz, CA), and rabbit polyclonal IgG against p-Rho A Antibody (Ser 188) (Santa Cruz Biotechnology®) followed by a 1:500 dilution of an Alexa Fluor 488-conjugated goat IgG anti-rabbit (Molecular probes, Life Technologies). Cells were counterstained by incubating with a 1:20 dilution of Rhodamine phalloidin (Molecular probes, Life Technologies) in PBS (pH7.4) and mounted with VECTASHIELD® mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). Epifluorescence microscopy was performed using a Nikon TE2000u and Metavue software. Confocal microscopy was performed using a Zeiss Axiovert 200M inverted microscope equipped with a Hamamatsu Orca camera. Images were obtained using the AxioVision software (Zeiss) employing the ApoTome structured light illumination system.
**Immunoblotting**

MDA-MB-231 cells/well were grown in 10cm dishes plate under low serum conditions (DMEM Hi-Glucose supplemented with 1%FBS, 1% Gentamicin) until they reached 70% confluence. Cells were then treated with 0.1% EtOH or 100 μg/mL *R. crenulata* for 24 hours. Protein from the treated cells was harvested by lysing the cells with 0.5mL RIPA per 5 x 10^6 cells for 30 minutes at 4°C. The cells were scraped with clean cell scrapers; the lysates were collected in 1.7mL microcentrifuge tubes, vortexed briefly, and passed five times through a 27½ gauge needle. The insoluble material was pelleted by centrifugation and the supernatant was collected. The protein was quantified via a BCA assay by the 96-well plate method (Thermo Scientific, Pierce). 30μg of protein were denatured by boiling with SDS sample buffer (50mM Tris Base (Trizma Base), 100mM Dithiothreitol, 0.1% v/v SDS and 10% Glycerol, final concentrations at pH 6.8) for five minutes.

Protein samples (50μg) were denatured in protein loading buffer (4% (w/v) SDS (sodium dodecyl sulfate; electrophoresis grade, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, and 200 mM DTT (dithiothreitol)) and separated via gel electrophoresis on a 12% polyacrylamide/bis-acrylamide (29:1) gel then transferred and immobilized onto a PVDF membrane. The proteins were blocked in 5% Non-fat dry milk dissolved in 5% TBS-T. The membranes were washed and incubated with antibodies against 1:1000 rabbit polyclonal IgG against RhoA (6739) (Cell Signaling), 1:1000 rabbit polyclonal IgG against ROCK1, and 1:5000 rabbit polyclonal IgG against β-Actin dissolved in blocking solution. Secondary antibodies conjugated to horse radish peroxidase (HRP; Santa Cruz
Biotechnologies) were used to detect the primary antibodies on the blots (1:1000 goat IgG against rabbit). All secondary antibodies were dissolved in blocking solution. Membranes were washed and incubated with chemiluminescent reagent (Santa Cruz Biotechnology®, Inc., Santa Cruz, CA). Images of the chemiluminescence were developed on Kodak Scientific Imaging film and subsequently scanned or were imaged in a G:BOX Chemi Gel Imaging System equipped with GeneSys automatic control software (SYNGENE, Frederick, MD)

**MTS Assay**

An MTS proliferation assay was done using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) following the manufacturer’s instructions. Briefly, cells were cultured in 96-well plates and treated as required for each experiment. After incubation with the desired treatment the cells were washed with PBS and incubated in 100μL of PBS. The MTS solution was thawed at 37°C for 10 minutes and 20μL of MTS solution were added to each well. The plate was incubated for 2 hours at 37°C in a humidified, 5% CO₂ atmosphere. The absorbance of the formazan product at 490nm was measured on a Victor³ microplate reader (Perkin Elmer, Waltham, MA).

**Xenopus laevis Eggs, Embryos and Tissue explants**

Xenopus laevis eggs were obtained, fertilized, cultured and staged according to Nieuwoop and Faber (1967) by Dominique Alfandari, Helene Cousin and Genevieve Abbruzzese. Embryo microinjections, neural crest explants and movies were also kindly done by members of the Alfandari laboratory as described in (Alfandari, Cousin et al. 2003).
Imaging of *Xenopus laevis* Embryos and Neural Crest Explants

Picture of embryos and explants were taken using the Nikon SMZ stereomicroscope, the Nikon D50 digital camera and the Nikon Capture Software. Embryonic and explant cells were observed using a Zeiss Axiovert 200M inverted microscope equipped with a Ludl xyz-stage control and a Hamamatsu Orca camera. Images were taken using either SPOT software or AxioVision software (Zeiss) using the ApoTome structured light illumination system (Zeiss), where applicable.

Cell Adhesion Assays

The adhesion assays were performed as described in (Gaultier, et al. 2002). Briefly, wells of an EIA/RIA 96-well plate (Cat No. 3590 , Corning®, Corning, NY) were coated with 100μL solution of the following solutions: 20μg/L bovine fibronectin (Sigma, Saint Louis, MO), 1X attachment factor (denatured collagen, Gibco, Carlsbad, CA), or Mouse laminin I (Rockland, Gilbertsville, PA) in PBS. Negative control wells were left untreated. The plates were incubated at either at 37°C for 4 hours in a humid atmosphere or overnight at 4°C in a humid chamber. The solutions were carefully aspirated from each well and replaced with 200μL of sterile of 3mg/mL bovine serum albumin (A9418) (BSA, Sigma, Saint Louis, MO) in PBS blocking solution. Plates were incubated for either 30 minutes at 37°C under a humid atmosphere or overnight at 4°C in a humidity chamber. Prior to the assay coated cell adhesion assay plates were allowed to reach room temperature. The BSA was removed immediately before performing the assay. To perform the assay cells were grown to become about 70% confluent and treated for a
defined period of time. The cells were then collected via trypsin treatment, re-suspended in fresh low serum treatment medium and seeded in the adhesion assay plate. Following a one hour incubation at 37°C, 5%CO2, under a humid atmosphere the assay plate was aspirated and washed thoroughly 3 to 5 times with PBS to remove unattached cells. The cells were then fixed by incubating with 3.7% formaldehyde in PBS or 4% paraformaldehyde in PBS for 10 min. After removal of the fixative the cells were incubated with 1.0% crystal violet solution for 10 minutes. The crystal violet was removed and the cells were washed with distilled water until the wash water was clear. The cells were then incubated with 0.1% SDS in PBS for 10 minutes on a rocker. The plates were briefly centrifuged to prevent bubble formation and the absorbance at 590nm of the dissolved crystal violet was measured for each well using a Victor³ microplate reader (Perkin Elmer, Waltham, MA).

**Isolation and identification of phytochemicals from the root of *Rhodiola crenulata* L. (Hook & Thomson, Ohba)**

**Reagents and instruments**

Reagents were purchased at the highest commercial quality and used without further purification unless otherwise stated. Crude Rhodiola (where did you get it from) extract was analyzed by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as visualizing agent and an ethanolic solution of anisaldehyde and heat as developing agents. Fractions were also monitored using Agilent 1100 series LC-MS with UV detection at 254 nm and a low resonance electrospray model (ESI). Purification of title compounds was accomplished by flash column chromatography using E. Merck silica gel (60, particle size 0.040–0.063mm) or
Biotage Isolera Four with reverse phase silica gel. 1H and 13C NMR spectra were recorded on a Bruker (AV-400 or DRX-500 MHz) NMR spectrometer instruments and calibrated using residual undeuterated solvent (CDCl3:  δH = 7.26 ppm, δD = 77.16 ppm; CD3OD:  δH = 3.31 ppm, δC = 49.00 ppm) as an internal reference. The following abbreviations were used to designate the multiplicities: s=singlet, d=doublet, t=triplet, q=quartet, quin=quintet, hept=heptet, m=multiplet, br=broad. High resolution mass spectra (HRMS) were recorded on an Agilent ESI-TOF (time of flight) mass spectrometer using MALDI (matrix-assisted laser desorption ionization) or ESI (electrospray ionization) or a Waters Xevo G2 Q-ToF mass spectrometer. Compounds were analyzed using electrospray ionization in positive-ion mode. Purity of final compounds was >95% based on analytical HPLC and NMR analysis.

Plant material

Red colored-fine powder Rhodiola crenulata (Lot # 980106, Barrington Chemical Corporation; 500 Mamoreck Avenue; Harrison, NY) was kept in the dark at 4 °C until ready for use.

Extraction and isolation

The Rhodiola crenulata (9.6 g) was extracted with a Soxhlet apparatus in refluxing isopropanol (250mLx2) for 24 hours. The mixture was filtered and the solvent was evaporated to afford crude red/brown syrup, which was purified by a reverse phase and the non-polar compounds re-purified via normal phase silica gel column chromatography. The major isolated components from this syrup were salidroside and isopentenyl 3-O-b-
D-glucopyranoside. Other less abundant components were isolated (discussed in Results section). All compounds were identified by 1D, 2D NMR and mass spectroscopy.

**Identified Compounds**

The identified structures are shown in the Results section of this manuscript. The chemical structures found in this report were built using ChemBioDraw Ultra and color coded for ease of distinction and aid in discussion. SMILES nomenclatures were obtained for each structure with ChemBioDraw Ultra and used to run a Structure Ensemble Approach (SEA, www.bkslab.org) analysis. The SEA relates proteins based on chemical similarities among their ligands and serves as a starting point to build cross-target similarity maps Keiser, Roth et al. (2007). I selected the most significant predicted interactions (lowest E-values) and report them in the Results section.

**Results**

MDA-MB-231 cells are a triple negative (estrogen receptor, progesterone receptor, Her2/Neu receptor negative), highly invasive cancer cells isolated from a human, malignant, mammary adenocarcinoma. The triple negative phenotype is one of the characteristics that render the MDA-MB-231 cells resistant to current receptor targeted chemo-therapeutic treatments. Our lab has previously shown that *R. crenulata* inhibits the capability of MDA-MB-231 cells to migrate and seal a scratch wound, and their capability of invasion, as tested in a trans-well assay (Tu, Roberts et al. (2008). Our group has previously reported that high concentrations of sera can interfere with the effects of *R. crenulata* on murine cancer cells (Tu, et al. 2008). I confirmed that treatment of MDA-MB-231 cells with the *R. crenulata* extract inhibits the capability of these cells
to migrate and seal a scratch wound in the presence of high and low amounts of sera (10% and 1% FBS, respectively) (Figure 1). Our group has also found that cells treated with *R. crenulata* detach with more difficulty from their culture substrate than ethanol treated cells (unpublished observations). To determine if the anti-migration effect of *R. crenulata* could be due to an increased attachment of the cells to the available substratum we pre-treated MDA-MB-231 cells with vehicle or *R. crenulata* for four hours and performed an adhesion assay. MDA-MB-231 cells treated with *R. crenulata* displayed increased attachment to fibronectin, collagen (denatured collagen in the form of gelatin) and laminin substrates as compared to their vehicle treated counterparts (Figure 1).

Because migration is an essential process of cancer invasion and embryo development we decided to look at the effect of *R. crenulata* treatment on developing *X. laevis* embryos a well-established animal model of embryonic development that was readily available to us via the Alfandari laboratory of the Department of Veterinary and Animal Sciences at the University of Massachusetts, Amherst. *X. laevis* also provided an opportunity to explore the effect of *R. crenulata* both *in vivo* and *in vitro* as cultured explants of neural crest cells display a consistent migratory behavior *in vitro* that closely mimics their behavior *in vivo* (Alfandari et al., 2003). We expected that, if *R. crenulata* inhibited cell migration embryos would fail to carry out normal morphogenetic movement including gastrulation, neural tube closure and neural crest cell migration resulting in abnormal antero/posterior axis development. Embryos were treated either before or after gastrulation to test if cell migration was affected during these phases. Embryos treated with 0.1% ethanol, our delivery control, developed normally, with no
discernible morphological differences to the untreated control embryos. On the other hand, we found that embryos treated with 100μg/mL of R. crenulata extract before gastrulation displayed multiple interesting phenotypes including a shorter antero/posterior axis, and a failure to close the neural tube despite the fact that in most cases the blastopore was closed indicating that gastrulation had progressed (Figure 2).

To test if R. crenulata affected normal embryonic cell migration, we cultured cranial neural crest cell explants (CNC) explants on fibronectin coated substrates with or without the drug. The test were performed either by adding the drug from the onset or after CNC had initiated migration for 4 hours. Control were treated with the vehicle (0.1% ethanol) Control cranial neural crest (CNC) cell explants exhibited a normal pattern of migration where they first attached to the substrate, migrate as a cohesive sheet of cells for 3 to 5 hours and then migrate as individual cells (data not shown, see Alfandari, et al., 2003). Morphologically the attachment of CNC cells to the substrate is observed as a region of less cellular density. Close examination of the area allows the identification of individual cells within the attached regions. These cells are distinct in their spread morphology, and distinguishable nucleus and cytoplasm, as shown in figure 5. The CNC cells divide into segments and spread as either cell sheets or individual cells. Ethanol treated cells displayed a similar behavior where the cells attached, segregated into segments and migrated in sheets and individually. R. crenulata treated cells, however, showed a dramatic reduction in cell migration when treated prior to the onset of migration (Figure 3). Additionally in long-term culture after 10 hours of migration, the individual cells detached from the substrate and became round (Figure 5). This is often observed in CNC
that are not free to migrate \textit{in vitro} and can either be due to cell death, or loss of cell adhesion. To further test how \textit{R. crenulata} interfere with cell migration we then added the drug after the onset of migration. We found that the progression of the cells was halted but cells remained spread without motility (Figure 5b). This suggests that while cell adhesion was not inhibited, cell migration was impaired suggesting that cytoskeleton dynamics may be altered in these cells.

To determine whether \textit{R. crenulata} also exerted a modulator effect in MDA-MB-231 cell GTPases I performed immunofluorescence to determine the intracellular localization of RhoA and its downstream effectors. I found that ROCK1 distribution was confined to the trailing edge and around dividing nuclei of the immunolabeled cells (Figure 4b). In contrast, cells treated with \textit{R. crenulata} displayed ubiquitous distribution of ROCK1 in cells with a migration-like morphology (Figure 4b). No other discernible changes in distribution were observed when we examined total intracellular protein levels of RhoA, phospho-RhoA (P-RhoA), or phosphorRac1/Cdc42 (P-Rac1/Cdc42) (data can be found in the Appendix).

To determine if \textit{R. crenulata} inhibits cell migration by altering the GTPase activity CNC cells were treated with either ethanol or \textit{R. crenulata} with and without the addition of the specific ROCK chemical inhibitor Y-27632 (Cytoskeleton Inc., Denver, CO). Cells responded to the ethanol and \textit{R. crenulata} treatments as described above (Figures 5a and 5c). We found that cells treated with ethanol and Y-27632 (Figure 5b) attach at a slightly slower rate than their ethanol-only treated counterparts, but they manage to divide into segments and migrate in sheets and individually. Additionally these cells looked flatter than the ethanol treated counterparts (Figure 5b and 5c, respectively). While cells treated
with *R. crenulata* (Figure 5c) showed inhibition of migratory behavior, cells treated with
*R. crenulata* and Y-27632 (Figure 5d) regained, at least partially, their capability to
spread and migrate. This effect is consistent with previous studies showing that elevated
ROCK activity-induced cell rounding can be reversed by treating cells with Y-27632
(Wilkinson, Paterson et al. (2005). The partial reversal of *R. crenulata* treatment by Y-
27632 suggests that the *R. crenulata* inhibits CNC migration, at least partially, by
increasing or enhancing ROCK signaling.

Given the mixture nature of the hydroalcoholic *R. crenulata* extract it is difficult to
pinpoint those processes affected by *R. crenulata* that confers the extract its anti-cancer
properties. In order to evaluate and develop *R. crenulata* as a chemopreventive or
chemotherapy its exact composition must be determined and each component requires
testing to identify its effect, if any, on oncogenic processes. To identify the active anti-
migration component (or components) of *R. crenulata* we sought to fractionate and
characterize the compound mixture. We confirmed the presence of previously described
*R. crenulata* compounds (Nakamura, Li et al. (2008), and identified various known
compounds in the *R. crenulata* root (Figure 6). Three of the isolates are mixtures of
known compounds and minute quantities of unidentified molecules, suggesting that these
fractions, if they display anti-cancer activity, might do so via a synergistic effect.

Once we identified most compounds of *R. crenulata* we sought to determine if any of
these compounds have anti-migration activity. To do so we tested the effect of each
compound or fraction on MDA-MB-231 cell migration in response to a scratch wound.
We treated the cells with the diluted compounds in low serum medium, performed a
scratch and took images of the scratch wound over time, for at least 48 hours. We then
determined the relative wound density over time to allow us to determine which compound delays wound closure and therefore would inhibit migration. Figure 7 summarizes our findings. We found that four compounds prevented proper wound closure as compared to delivery controls: FRHB1-163D, FRHB1-1069, FRHB1-1071, and FRHB1-1072. Of these FRHB1-1071 and -1069 displayed anti-migration activities that closely mimics that of *R. crenulata*. FRHB1-163D, FRHB1-1072 and TA3 (assay control), a molecule similar to the HIF-1 inhibitor, EF24, displayed anti-migratory activity higher than that of *R. crenulata*. Of these FRHB1-1071 and -1072 are mixtures containing different ratios of known compounds and at least two unknown compounds, as determined by thin layer chromatography (TLC). These compounds are found in such small quantities that their isolation has been difficult and will require further efforts for their characterization to be completed. Upon completion we expect to test the compounds and determine their anti-cancer activity.

**Discussion**

We have shown that *R. crenulata* inhibits migration and invasion of cancer cells. It is likely that the extract inhibits migration and invasion by disrupting the mechanisms involved in remodeling of the actin cytoskeleton fibers, and we have determined that part of the disruptive effect of *R. crenulata* on cell adhesion and migration is due to modulation of the RhoA GTPase effector ROCK1. Gadea, et al. (2007) have shown that loss of p53 results in activation of the RhoA-ROCK pathway and leads to ameboid migration of mouse embryonic fibroblasts (MEFs) and melanoma cells. We have previously found that p53 might be deregulated in invasive cells treated with *R. crenulata*. Microarray analysis showed that p53 mRNA transcript levels decreased after
24 hours of *R. crenulata* treatment (see pathway figure in Appendix). However, verification of this trend was not explored. We previously investigated if p53 was somehow affected by checking mRNA expression levels upon vehicle or *R. crenulata* treatment in cell lines other than 76NTert-ssiSFRP1, but no change were observed. At the time, however, we did not explore the activity levels of p53 and p53 mutants (data not shown). It is possible that *R. crenulata* affects p53 activity and this change might result in ROCK localization or activity deregulation. Based on the findings by Gadea, et al. (2007) it would seem counterintuitive to hypothesize that a decrease of p53 caused by *R. crenulata* would halt cell migration. However, given that multiple compounds are found in the *R. crenulata* extract, and each compound potentially has a distinct activity, it is not possible to state with certainty whether the interactions between ROCK1 and p53 are affected by *R. crenulata*, and thus require further exploration.

A possibility exists that the delocalization of ROCK1 and the “degree” of activity it possesses after *R. crenulata* treatment might be sufficient to stop the progress of migration. Besides effecting the assembly of the actin cytoskeleton and its contractility, the positioning of GTPases such as Cdc42 and RhoA determine the asymmetry that gives rise to the leading and trailing edges of polarized migrating cells. For example, at the leading edge, Liver Kinase B 1 (LKB1) binds to active Cdc42 and p21 Activated Kinase (PAK) in motile cells. This association allows for the induction of phosphorylation (and activation) of PAK by active Cdc42 (Zhang, Schafer-Hales et al. (2008)). These events take place at the leading edge of the cells and results in the recruitment and activation of the Par6-aPKC complex and this activation triggers a signaling cascade that results in the release of adenomatous polyposis coli (APC) which then binds to microtubules (MTs),
anchoring them to the cell membrane. The anchorage of the MTs to the membrane promotes polarization of the cytoskeleton, re-localization of the MT organizing center (MTOC), Golgi and other cellular components towards the leading edge, providing materials for further membrane protrusions. Additionally, the Par6-aPKC complex targets other proteins that degrade RhoA (Aranda, Nolan et al. 2008), amongst them the ubiquitin ligase Smurf1 (Wang, Zhang et al. 2003). The entire process results in a gradient of distribution of polarization proteins and the resulting polarized cellular movements. Given the fine tuning orchestration of the processes that permit migration to take place, it is likely that disruption of ROCK1 localization may prevent cell migration. Essentially, cells treated with *R. crenulata* would be “stuck” to an area, incapable of polarizing and moving with directionality.

In summary, our data indicates that *R. crenulata* may inhibit cancer cell migration by interfering with actin cytoskeleton dependent invadopodia. Cell directionality was studied by Matthews, Carmona-Fontaine, et al. (2008) both *in vitro* and *in vivo*. Matthews and colleagues showed that migration of neural crest cells (NCC) in both *Xenopus sp.* and zebrafish is controlled by Syn-4 and the non-canonical Wnt planar cell polarity (PCP) pathway, modulated by Disheveled (Dsh), and is controlled by the RhoA pathway. It would seem that *R. crenulata* increases the intracellular levels of ROCK1 in migratory cells, thus inducing cytoskeletal changes that disrupt cell polarity and hinder the cancer, or embryonic cells’, capability to migrate. The results of this project are sure to provide further insight into the role of migration regulators in development and disease.
Figure 11. *R. crenulata* inhibits the migration capability of triple negative breast cancer cells and increases their capability to attach to various substrata. *R. crenulata* inhibits MDA-MB-231 migration in both low (1\% (A)) and high (10\% (B)) concentrations of serum. (C) Treatment of invasive cells with 100\μg/mL *R. crenulata* increased the attachment of the invasive cells to fibronectin, laminin, and collagen substrata. The graph shows the representative results from one of seven assays. Each experiment included four replicas of each treatment and substratum conditions.
Figure 12. Treatment of *Xenopus laevis* embryos during early or late gastrulation interferes with embryonic development. Top: *X. laevis* embryos were treated before gastrulation with 0.1% ethanol (EtOH: vehicle) (mean 50 embryos per replica), 100 μg/mL *R. crenulata* (mean 61 embryos per replica), or left untreated (mean 54 embryos per replica) and allowed to develop until the neural tube closure stage when they were examined (3 replicates performed). Three separate sets of *X. laevis* embryos were treated after gastrulation had initiated with vehicle (mean 26 embryos per replica), 100 μg/mL *R. crenulata* (mean 25 embryos per replica), or left untreated (mean 25 embryos per replica) and allowed to develop until the neural tube closure stage when they were examined (3 replicates performed). After thirty hours of incubation a final count of the embryos was performed. The mean number of live embryos per group (after a 30 hour incubation) is indicated at the bottom right corner of each representative image. *Spina bifida* morphology (bottom): The pre-gastrulation embryos treated with 100 μg/mL *R. crenulata* that survived were allowed to develop for an additional 72 hrs. Upon examination a *spina bifida*-like phenotype was observed. Controls are absent because they were discarded prior to discovery of the phenotype. (White arrows indicate areas where neural tube failed to close. Yellow arrows indicate regions where partial neural tube closure has ensued). Post-gastrulation treated embryos with *R. crenulata* died.
**Evaluates cranial neural crest (CNC) migration.** CNC explants were incubated on fibronectin substrate in Danilchik’s media (with 1 mg/ml sulphate) (Alfandari et al, 2003) with 0.1% ethanol (top) or 100µg/mL, R. crenulata (bottom). A subset of CNCs was left control. Time-lapse images were taken (time points represent hours). EtOH treated explants attach and spread, but *R. crenulata* images were taken at 4X magnification over a period of 12 hours. Numbers on each representative images correspond to hours of
-MB-231 cells with the *R. crenulata* extract increases cell adhesion on all substrates tested possibly by altering the migrating cells. (A) Cells were treated with *R. crenulata* or vehicle for the indicated time periods. Total protein was harvested and to determine if levels of RhoA and its downstream targets were affected by either treatment. No significant changes in protein for a potential slight increase in phosphorylated RhoA (P-RhoA) after 2 hours of treatment and the levels of RhoA and ROCK1 however, the changes were slight and the β-Actin loading control signal was very strong, such that the protein level changes were not apparent. (B) Immunocytochemistry against ROCK1. Cells were treated with vehicle or *R. crenulata* for 4 hours. Nuclei were stained with DAPI (blue), actin fibers were stained with Texas Red–phalloidin (red). (C) MDA-MB-231 cells were treated for 12 hours with either vehicle or RhoA inhibitor Y-27632 at a final concentration of 10 μM. Prior to assay crystal violet dissolution step (Columns from left to right: BSA, fibronectin (Fn), laminin (Ln), and attachment to tissue culture plates). The graph represents one of two assays done with 8 replicates per condition, per assay (n=16, mean ± SEM.).
Figure 15. Time lapse brightfield microscopy of CNCs treated with ethanol or *R. crenulata*, each with or without the specific chemical ROCK inhibitor Y-27632. CNCs were cultured on fibronectin coated plates and allowed to adhere to the substrate (4 hours). Time-lapse images were taken after the treatments were added, over a ten hour period (numbers indicate total hours of CNC culture). (A) CNCs treated with 0.1% EtOH spread normally (as compared to untreated CNCs (data not shown)). (B) We observed that cells treated with EtOH and Y037632 spread faster than the EtOH counterparts and showed a more spread morphology. (C) Cells treated with *R. crenulata* displayed impaired migration and a rounded morphology. (D) When *R. crenulata* treated cells were concomitantly exposed to Y-27632 we observed a partial rescue in cell spread and migration. (Images in panels A-D were taken with 10X magnification) (E) Close-ups of all treatments at time-points 4 hours apart show the spread morphology of cells in EtOH treated specimens as compared to the rounded morphology observed in *R. crenulata* treated cells. The last panel clearly shows partial rescue of the spread phenotype when cells treated with *R. crenulata* were exposed to the ROCK inhibitor Y-27632. Images are representative of two experiments done with four replicas each.
MDA-MB-231 FRHB1 Multiple Compounds Anti-migration Screen (20uM)
Fluorescent images of various cytoskeletal regulating proteins, and adhesion-related proteins were done to determine if *R. crenulata* had an effect of intracellular protein distribution. We found no discernible differences between ethanol and *R. crenulata* treated cells. The experiments were performed three times, each time in duplicate, as described for Rock1 immunostaining. Representative images of each experiment were selected and are shown here. (EtOH – ethanol; Tx – treatment; SW – scratch-wound; h – hour)
Figure 18. Fluorescent images of various cytoskeletal regulating proteins, and adhesion-related proteins were done to determine if \textit{R. crenulata} had an effect of intracellular protein distribution. We found no discernible differences between ethanol and \textit{R. crenulata} treated cells. The experiments were performed three times, each time in duplicate, as described for Rock1 immunostaining. Representative images of each experiment were selected and are shown here. (EtOH – ethanol; Tx – treatment; SW – scratch-wound; h – hour)
Images of various cytoskeletal regulating proteins, and adhesion-related proteins were done to determine if *R. crenulata* affected intracellular protein distribution. We found no discernible differences between ethanol and *R. crenulata* treatments were performed three times, each time in duplicate, as described for Rock1 immunostaining. Images from each experiment were selected and are shown here. (EtOH – ethanol; Tx – treatment; SW – scratch-wound;
Regulating genes affected in 76NTert-siFRP1 cells after treatment with *R. cremulata* as compared to the control. Microarray data analysis using the Ariadne P6 software (as described in chapter 2) revealed changes in expression levels of various genes, such as p53 (green circle). Red indicates an up-regulated target, gray indicated no change, and blue indicated down-regulation of the target transcripts. The shades of color allow for comparison of the relative transcript changes.
Figure 21. The scheme represents the proposed mechanism by which *R. crenulata* disrupts cell polarity, adherence and, subsequently, migration. We propose that disruption of the intracellular levels and the localization of cytoskeleton modulating proteins by *R. crenulata* results in random anchorage and release foci and that such foci disarray results in concomitant attachment and detachment of the cells and lack of directionality, effectively anchoring the cells to one locus.
References


Nieuwkoop, Pieter D. (Pieter Dirk) & Faber, Jacob (1967). Normal table of Xenopus laevis (Daudin).: A systematical and chronologcal survey of the development from the fertilized egg till the end of metamorphosis. Hubrecht-Laboratorium (Embryologisch Instituut)
CHAPTER 4

EFFECTS OF R. CRENU LATA ON CANCER CELL METABOLISM

Metabolism and Cancer

Alterations in cellular metabolism have been suggested as contributors to the development of cancer for over 50 years. Perhaps the best known proponent of such phenomenon was Otto Warburg. On May 1955 Otto Warburg delivered a lecture at Stuttgart before the German Committee for Cancer Control. The lecture highlighted the higher rates of fermentation displayed by cancer cells as opposed to their normal cell counterparts. Warburg did numerous detailed measurements of cellular respiration and fermentation and noticed that there was “one common cause into which all other causes of cancer merge, the irreversible injuring of respiration” (Warburg, 1956). At that time it was known that respiration took place mostly in the cell grana (now known as mitochondria) and fermentation took place in the cytosol. Warburg emphasized that the fermentation by body cells was greatest during early embryo development and that it decreased gradually over the course of embryonic development. He considered that cancer developed when dedifferentiation of the body cells occurred, resulting in injury of respiration and increased fermentation. Because the increase in fermentation occurs when there is still some respiration occurring, Warburg (Warburg, 1956) concluded that a transitional phase must be achieved “where fermentation has become so great that de-differentiation has commenced, but not so great that the respiratory defect has been fully compensated for energetically by fermentation, we may have cells which indeed look like cancer cells but are still energetically insufficient [and] such cells have been referred to as “sleeping cancer cells”, which,
nowadays, we have possibly named “cancer progenitor cells”, or “cancer stem cells”. Warburg also stated that “from this point of view, [the terms] mutation and carcinogenic agent are not alternatives, but empty words, unless metabolically explained”. Many of his findings regarding changes in cellular metabolism still hold true today.

The term “aerobic glycolysis” is commonly used to describe the elevated lactate production by tumors or proliferative tissues using glucose even in the presence of plenty oxygen. This is also referred as the “Warburg effect”, which is thought to confer survival advantages to cancer cells. Recently, Pavlides et al. (2009) proposed that cancer epithelial cells induce the Warburg effect in neighboring stromal fibroblasts. These cancer-associated fibroblasts undergo myo-fibroblastic differentiation, display increased glycolysis markers, and secrete lactate and pyruvate, the products of aerobic glycolysis. Additionally they reported that the cells that underwent myofibroblastic differentiation up-regulated markers of oxidative stress, suggesting that the Warburg effect is accompanied by over-production of reactive oxygen species (ROS). Increased ROS production in cancer cells can cause DNA damage leading to genome instability and mutations (Hanahan & Weinberg, 2011). Genetic instability can enable a cell to acquire phenotypic hallmarks of cancer, such as sustained proliferative signaling, the evasion of growth suppressors, the activation of invasion and metastasis, and replicative immortality among others (Hanahan & Weinberg, 2011). There is evidence to support that high levels of ROS induce and maintain senescence-induced tumor suppression (Courtois-Cox, Jones, & Cichowski, 2008). ROS are also implicated in the enhancement of tumor development, sustainment of the phenotypic hallmarks of cancer including the Warburg effect, and cancer cell growth.

The mechanisms by which ROS act as chemo- preventative and anti-cancer agents are numerous, including the induction of apoptosis (Fruehau & Meyskens, 2007; Oyagbemi, Saba,
& Ibraheem, 2009; Thannickal & Fanburg, 2000; Trachootham, Alexandre, & Huang, 2009), inhibition of cell proliferation (Vincent & Crozatier, 2010), activation of DNA repair pathways (Kuilman, Michaloglou, Mooi, & Peeper, 2010; Ray, Huang, & Tsuji, 2012), and the suppression of protein synthesis and increased autophagy (Ray et al., 2012). Because ROS regulate a plethora of cell signaling pathways necessary for cell survival, immunity, and cell differentiation, the deregulation of ROS homeostasis can also lead to cell death. Normal cellular growth and metabolism are reliant upon the fine-tuned balance of intra- and extra-cellular ROS levels. For example, an increase in ROS triggers premature differentiation of hematopoietic progenitors into three mature blood cell lines in Drosophila larvae via activation of FoxO and Jnk pathways and down-regulation of Polycomb (Fruehau and Meyskens 2007Fruehau). If tumor ROS overcome a certain threshold cell death ensues, usually via apoptosis (Trachootham et al. 2009). Nevertheless, evidence presented by Ishikawa, et al., (2008), has drawn a relationship between mitochondrial ROS and tumor aggressiveness. They found that pretreatment of highly metastatic cancer cells with ROS scavengers suppressed their metastatic potential in vivo (Ishikawa et al., 2008). Though our understanding of the role of ROS in cell health and metabolism is expanding, clear relationships between ROS and cancer are yet undefined. This lack of information hinders the development of therapies based in ROS modulation.

Natural products have been the main source to approximately 70% of the FDA approved anti-cancer drugs developed from the 1940s to 2010 (Newman & Cragg, 2012). Studies have shown that dietary antioxidants derived from natural products take part in cellular redox reactions, acting as antioxidants or pro-oxidants (Oyagbemi et al., 2009). Some known anti-cancer natural products include resveratrol (Jang et al., 1997), curcumin (Thomas et al., 2008), and silvestrol and pancratistatin among others (Kinghorn, Chin, & Swanson, 2009). Recently, McAllister and
colleagues (McAllister, Christian, Horowitz, Garcia, & Desprez, 2007) demonstrated that treatment of malignant breast cancer cells with the natural product cannabidiol (CBD) activated ERK signaling pathway, down-regulated Id-1 to almost undetectable levels, up-regulated Id-2, and increased ROS production. CBD also inhibited mouse mammary cancer cells proliferation and their ability to invade other tissues and reduced primary tumor volume and the number of lung metastatic foci in vivo (McAllister et al., 2011). Given the previous reports it is reasonable to suggest that abrogation or disruption of ROS homeostasis in cancer cells can lead to cancer cell death.

**Relationship between metabolism and migration**

Changes in metabolism can affect invasive behavior of cancer cells by altering key signaling pathways such as RhoA-mediated cell adhesion. A clear relationship between RhoA-regulated cell-substratum adhesion and ATP levels has been found. Rho GTPases are responsible for tight junction assembly and maintenance. It has been shown that antimycin A (a respiratory chain inhibitor) induced-ATP depletion results in Rho inhibition and the disassembly or dysfunction of tight junctions in Madin Darby canine kidney (MDCK) cells (Gopalakrishnan, Raman, Atkinson, & Marrs, 1998). Further evidence of the regulatory role of ATP on Rho GTPases came from the work of Prahalad and colleagues (2004) who found that MDCK cells detach from collagen following glucose deprivation and then re-attach when glucose is replenished. The group found that depletion of ATP reserves in MDCK cells inactivated RhoA, which helped explain the loss of cell-substrate attachments. Interestingly, chemical inhibition of the Rho effector ROCK did not prevent glucose replenishment-related cell reattachment. Nevertheless, inhibition of ROCK prevented the formation of actin stress fibers or adhesion foci (Gopalakrishnan et al. 1998).
Treatment of the ATP-depleted, detached MDCK cells with ML-7, a myosin light chain kinase (MLCK) inhibitor, prevented the reattachment of MDCK cells (Gopalakrishnan et al., 1998; Prahalad et al., 2004). These observations suggest that ATP levels affect RhoA function and disrupt Rho-signaling, thus effectively delineating a link between cellular energetics and cytoskeletal regulation.

Further evidence supports the Rho-modulating role of ATP. When subjected to ATP depletion MDCK cells expressing constitutively active RhoA retained more peripheral actin than controls and exhibited complex networks of short actin in the interior of the cell’s basal surface. Constitutively active RhoA also prevented cell detachment induced by glucose deprivation (Gopalakrishnan et al., 1998; Prahalad et al., 2004). Therefore it is reasonable to conclude that a cell’s metabolism can control cellular migration by regulating ATP levels.

**Rhodiola crenulata and metabolism**

*R. crenulata* influences several aspects of metabolism. Feeding of *R. crenulata* decreased plasma insulin concentrations in Zucker Diabetic Fatty (ZDF) rats under fasting conditions without affecting plasma glucose concentrations (J. Wang et al., 2012). Additionally in ZDF rats subjected to an oral glucose tolerance test (OGTT), the *R. crenulata* decreased both glucose and insulin plasma concentrations. *R. crenulata* also lowered liver and plasma triglycerides and adipose insulin resistance and it accelerated the decline of non-esterified fatty acids (NEFA) in ZDF rats (Wang, Liu, & Huang, 2012). There is indirect evidence indicating that by modulating the pentose phosphate pathway (PPP) *R. crenulata* may inhibit adipogenesis (Lee, Kwon, Apostolidis, Shetty, & Kim, 2011). Thus, the beneficial effects of *R. crenulata* in prevention and treatment of cancer may be linked with its ability to alter metabolism.
We hypothesized that (a) the treatment of breast cancer cells with *R. crenulata* results in cell death due to a decrease in aerobic glycolysis, which is up-regulated in cancer cells; and (b) treatment with *R. crenulata* may cause stress to cancer cells beyond their metabolic thresholds that cannot be tolerated by metastatic cells resulting in starvation and subsequent cell death. In this chapter, preliminary data suggesting that *R. crenulata* indeed modulates metabolism of invasive cells is presented. Specific *R. crenulata* compounds could potentially be involved in metabolic regulation. While the initial data obtained were intriguing, a more detailed analysis as separate study is required to determine the potential mechanism(s) by which *R. crenulata* regulates metabolism and exerts its anti-cancer effects.

**Materials and Methods**

**ROS measurements with H$_2$DCFDA**

76NTert-siSFRP1, 76NTert-pSUPER and MDA-MB-231 cells were seeded in 96 well plates at a density of $1 \times 10^5$ cells/well in phenol red-free DMEM. Cells were treated with 2$\mu$M Calcein-AM or 5$\mu$M H$_2$DCFDA for 30 minutes at 37°C. Cells were subsequently treated with a 1:100 dilution of vehicle (10% EtOH) or *R. crenulata* extract (10mg/mL). A Perkin-Elmer Victor$^3$ microplate reader was used to measure the relative fluorescence in each well at various time-points. Ex: 490/20 CW-Filter and Em: 515 Slide filter were used for Calcein AM and carboxy-H$_2$DCFDA respectively. An increase in Calcein-AM intensity occurs when the permeable dye enters live cells and is cleaved by a live cell’s esterases into a non-permeable product which fluoresces. Therefore, in live cells, as the fluorophore accumulates in the cells so does the fluorescence intensity. Similarly, carboxy-H$_2$DCFDA fluorescence intensity increases following intracellular cleavage and oxidation.
Measurements of oxygen consumption rate (OCR or respiration)

Seeding of cells for XF24 assays

MDA-MB-231 cells were grown in DMEM High Glucose supplemented with 20μg/mL Gentamicin (6.25-fold less than the minimum concentration required to exert influence on cell growth and metabolism (Fischer, 1975) and 10% FBS. During treatments only 1% FBS was used in the medium. Cells were seeded at the density of 1 x 10^4 cells/well in 250μL medium in 24-well Seahorse V7 PS culture plates following the 2-step protocol. Wells A1, B4, C3, and D6 were kept empty to be used as blanks for temperature correction. Cells were incubated overnight at 37°C under 5% CO₂ to allow adherence to the culture dish and then examined for roughly even spread across wells. Treatments with 0.1% EtOH and 50μg/mL R. crenulata (final concentrations) were performed for 24 or 48 hours (five well replicates for each treatment group).

Preparation of the Cell Plate for XF24 Assay

Cells in the XF24 cell culture plate were inspected under microscope to assure confluence and even seeding. 150μL of growth media were removed from each well, being careful not to touch pipette tip to the bottom of the wells. Approximately 100μL of growth media were left behind in each well. The cells were rinsed with 1mL of pre-warmed low potassium buffer (LKB) (350μM KCl, 130μM CaCl₂·H₂O, 1μM KH₂PO₄, 120μM Na₂SO₄, 200μM MgCl₂, 0.2% BSA,
pH 7.4). Cells were incubated in 0.5mL LKB at 37°C incubator without CO2 for ~60 minutes prior to use.

**Loading of the desired compounds for injections/additions**

The XF24 Sensor Cartridge has designated Ports A, B, C, and D for additions of desired compounds. They are loaded prior to putting the cartridge for calibration. Each port was loaded with 50μL of pre-warmed (37°C) FCCP dissolved in respiration buffer. Unless otherwise indicated Oligomycin (2μg/mL), FCCP (2μM), Rotenone (1μM) and Antimycin A (1μg/mL) were used at indicated concentrations. There order of injection is described elsewhere e.g. within figure legends. The drug concentrations were empirically determined by titrations (not shown). Cells were treated with *R. crenulata* extract or ethanol control either prior to or during the experiment as indicated.

**Calibration of the XF24 Analyzer’s sensors and measurements**

Calibration of the XF24 Analyzer sensors was performed as per the manufacturer’s instructions. Cartridges were hydrated at least 12 hour in 1.0 mL of the Calibrant solution (pH 7.4). The sensor cartridge was placed on top of a 24-well plate containing Calibrant solution and incubated at 37°C in a non CO2 incubator overnight. The calibration was performed before assay for ~20 min. Before using the sensor cartridge desired compounds were preloaded in each port (A, B, C, D) and the calibration step was included in the experimental template. Measurements using cell plates were made only if the calibration of the sensors was successful based on the in-
built criteria of XF Analyzer software. The calibration of the biosensors employed in the extracellular flux (XF) bioenergetics assay and the mechanics of the assay are explained extensively in the report by Wu, et al. (2007). Repeated cycles of 1 min mixing, 1 min wait and at least 3 minute measurements were used, over the course of 120 minutes.

**Fluorescence Immunocytochemistry**

1x10^5 MDA-MB-231 cells were seeded in 24-well plate containing culture grade cover-glasses and cultured in their corresponding media. Cells were treated with vehicle (0.1% ethanol) or 100µg/mL *R. crenulata* extract and incubated at 37°C under 5% CO₂. 24 hours post-treatment, cells were fixed and permeabilized by incubating with 0.1% Triton X-100 in PBS for 10 min. at 4°C. Cells were blocked with blocking buffer (containing F(ab')2 and 20% goat serum) and incubated with a 1:500 dilution of primary mouse monoclonal anti-human p62 IgG antibody (Abcam) in blocking buffer for 2 hr at room temperature. After primary antibody removal and three washes with PBS, the cells were incubated with an Alexa Fluor® 594-conjugated secondary goat anti-mouse IgG (Invitrogen) antibody at a 1:500 in blocking buffer for 30 minutes at room temperature. Finally, cells were washed and mounted with DAPI-containing mounting medium ( Vectashield, Vector Labs), sealed with toluene-free nail polish and observed using Nikon TE2000u fluorescence microscope and the Metavue software.

**Similarity Ensemble Approach**

The Similarity ensemble approach (SEA) is a free-of-charge bioinformatics tool that allows the user to relate proteins based on the chemical similarity (the chemical topology or three
dimensional surface and physicochemical properties of particles of two or more ligands). The SEA was used to search large compound databases and to build cross-target similarity maps in a chemo-centric fashion that reveals biological clusters of potential target proteins (Keiser et al., 2007). We used each compound's Simplified Molecular Input Entry System (SMILES™), a chemical language in which molecules and reactions are specified using ASCII characters, and a one-word identifier to run the SEA search. Compound SMILES were obtained with ChemBioDraw software (PerkinElmer, Cambridge, MA). The SEA search tool can be found at http://sea.bkslab.org. SEA is a public service site provided by the Shoichet Laboratory in the Department of Pharmaceutical Chemistry at the University of California, San Francisco (UCSF).

**Results**

*R. crenulata* effects on ROS metabolism

A significant change in ROS production, be it an increase or a decrease, can result in inhibition of cancer cell migration and metastasis or cancer cell death. Because the natural product CBD induced increase of ROS was found to inhibit cell migration and proliferation, we reasoned whether *R. crenulata* could exert similar effects by altering ROS levels in cancer cells. Therefore, we measured changes in the ROS levels in *R. crenulata* treated cells compared to controls (vehicle-treated and untreated). Our results show that after 24 hours of treatment with *R. crenulata*, 76NTert-siSFRP1 cells have higher levels of intracellular ROS compared to controls (*Figure 1*). The increase in ROS was similar whether the 76NTert-siSFRP1 cells were grown on a tissue-culture adherent-treated surface (*Figure 1c*) or ultra-low attachment surface (*Figure 1d*). The ROS increase in 76NTert-siSFRP1 (invasive cancer) cells was inversely proportional to cell
survival (Figure 2c,d). Interestingly, a decrease in ROS was observed in MDA-MB-231 (non-invasive cancer) cells (Figure 1a,b) and in control 76NTert-pSUPER (non-tumorigenic) cells treated with R. crenulata (Figure 1e,f) when compared to ethanol treated and untreated groups. It is not clear if the observed decrease in 76NTert-pSUPER and MDA-MB-231 ROS is the result of R. crenulata-induced cell death. The live MDA-MB-231 cell population seemed to decline after treatment with R. crenulata (Figure 2e,f).

_R. crenulata effects on the metabolism of MDA-MB-231 cells_

To determine if _R. crenulata_ alters cell metabolism, real-time metabolic assays were performed using the Extracellular Flux Analyzer (Seahorse Biosciences, Chicopee, MA), which permits simultaneous measurements of glycolysis (reported as extracellular acidification rate, ECAR) and oxidative metabolism (reported as oxygen consumption rate, OCR). Cells were treated with 0.05% ethanol or 50μg/mL _R. crenulata_ for two hours. Cells were then changed into XF Analysis buffer. ECAR and OCR were measured in a low buffered medium as described in the materials and methods section. After measuring the basal rates of OCR and ECAR, cells were successively treated with oligomycin (an ATPase or Complex V inhibitor), trifluorocarbonylcyanide phenylhydrazone (FCCP, a protonophore), rotenone (a Complex I or NADH-ubiquinone oxidoreductase inhibitor), and antimycin A (a Complex III or cytochrome c1 complex inhibitor). While the oligomycin-sensitive respiration gives an estimate of ATP-synthesis/turndover supporting respiration, the oligomycin insensitive respiration gives an estimate of the proton leak across the inner mitochondrial membrane (after subtracting the non-mitochondrial respiration that is insensitive to the successive addition of rotenone and antimycin A). The use of FCCP was expected to give an estimate of the maximal respiration.
Invasive cells treated with 50µg/mL *R. crenulata* for two hours prior to metabolic parameter analysis showed lower respiratory activity as compared to untreated and 0.05% ethanol-treated controls (*Figure 3a*). Lower respiratory activity in *R. crenulata* treated cells could be due to the inhibitory effect of the extract on cell growth. As expected, upon treatment with oligomycin the respiration of all groups dropped significantly. However, when the basal respiration was set to 100% to correct for differences among groups due to cell density/protein content, no significant difference in oligomycin-sensitive respiration was noted. Although in all groups FCCP addition increased respiration, it was still significantly lower than basal respiration. This suggests that under the experimental conditions used (i.e. 15 mM glucose as carbon source), when mitochondrial ATP synthesis is blocked, the respiratory activity of MDA-MB-231 cells is restricted. Most of the cellular respiration was rotenone-sensitive, and did not further decline upon antimycin A inhibition. This suggests that most of the respiratory activity in MDA-MB-231 cells is dependent upon oxidation of the Complex I substrate, NADH, in mitochondria.

Cells treated with *R. crenulata* showed higher ECAR values as compared to the ethanol treated and untreated cells (*Figure 4a*). This suggests a relatively higher rate of glycolytic activity, which may potentially decrease mitochondrial respiration as observed in the extract-treated cells. As expected, upon treatment with oligomycin, the degrees of increase in ECAR values were similar in all groups (*Figure 4B*). This suggests that the cells responded similarly to a block in the mitochondrial ATP synthesis regardless of the treatment received. This switch toward glycolysis has been previously observed, and can support ATP demand of cells in the absence of oxidative phosphorylation (OxPhos) (Kauppinen, McMahon, & Nicholls, 1988). A lack of further increase in ECAR values following the addition of FCCP suggests a potential limitation of mitochondrial pyruvate that would support the tricarboxylic acid (TCA) cycle and
CO₂-mediated acidification. No further increases in ECAR were observed with additions of rotenone and antimycin A. Measurements of OCR and ECAR values, normalized by the protein content (cell equivalents) in each assay group, did not show significant differences from the controls suggesting that the extract-treated cells responded similarly to respiratory chain inhibitors.

Chen, et al., (2007) have previously shown that inhibition of complexes I and II induce autophagy-mediated cell death in transformed and cancer cells in which ROS played a key role. Furthermore, J. Wu et al. (2009) have also shown that in mice that lacked Atg7, which is required for autophagosome formation in their skeletal muscles, have defective mitochondrial respiration. Cells from these mice showed a compensatory increase in glycolysis. Therefore, it is possible that the increase in glycolysis observed in R. crenulata treated cells could be due to reduced mitochondrial respiration resulting from the accumulation of damaged mitochondrial by impaired autophagy.

To help understand how R. crenulata may exert metabolic effects, we performed SEA analysis on isolated R. crenulata compounds (see chapter 3 for more details). Table 1 shows that at least six of the known compounds can potentially affect various metabolism related proteins. Only the relationships with the highest significance (lowest E-value) are included. The SEA analysis, which has been described by Keiser et al. (2007), found protein ligand relationships that concur with a family of proteins known to be modulated by R. crenulata treatment, (Doerner, 2007), for example cyclooxygenase 1 (COX-1). Given the predictive nature of the analysis further studies should be done to determine if the compounds listed in Table 1 are indeed R. crenulata targets. It will be interesting to validate the interaction between the compounds and
Complex I subunit, the NADH-ubiquinone oxidoreductase 4L, which may result in inhibition of Complex I and thus reduced respiration.

*R. crenulata effects on autophagy*

When cells experience nutritional starvation they undergo autophagy, a process that generates carbon and nitrogen sources by degrading cytoplasmic constituents. We reasoned that the beneficial effects of *R. crenulata* may be linked with its ability to influence autophagy in cancer cells. Therefore, we investigated if *R. crenulata* induced autophagy by monitoring changes in p62 localization/levels, a marker for autophagy. Foci of p62 proteins near the nucleus in response to *R. crenulata* treatment were observed, which is indicative of autophagy induction (Mizushima, Yoshimori, & Levine, 2010). The p62 foci were noted only in invasive cancer cells (*Figure 5*). Note that the p62 foci formed around the nucleus in 76NTert-siSFRP1 (invasive cancer) cells treated with *R. crenulata* (*Figure 5g, arrow heads*). However, the non-cancer cell line treated with ethanol displayed higher levels of p62 without prominent foci. This may be associated with its ability to interact with proteasome. The expression of p62 is induced upon proteasome inhibition (Bardag-Gorce, 2010; Sridhar, Botbol, Macian, & Cuervo, 2012). Ethanol is known to inhibit the ubiquitin-proteasome pathway, which may be responsible for the observed increase of p62 (French et al., 2001). Even though the concentration of ethanol used in this study had no discernible effect on cellular metabolism, it could be sufficient to induce p62 levels by inhibiting the proteasome. A possible depletion of nutrients/metabolites in cancer cells by *R. crenulata* could be the underlying cause of autophagy. Dando, et al. (2013) found that natural product-derived cannabinoids increase AMP/ATP ratios via ROS, attenuating glycolysis. The group also found that PANC1 cells (epitheloid pancreatic carcinoma cells) treated with
cannabinoids display increased AMPK phosphorylation levels and undergo AMPK-induced autophagic cell death (Dando et al., 2013). Given previous reports and our data, it is reasonable to suggest that *R. crenulata*’s beneficial effects may be linked with the induction of autophagy in cancer cells. Nevertheless, the effects of *R. crenulata* on cancer metabolism remain to be further dissected *in vitro* and *in vivo*.

**Discussion**

The importance of altered metabolism in cancer development has been recognized for over five decades. Therefore, targeting the metabolism of cancer cells without altering the metabolism of normal cells is an attractive strategy for cancer therapy. The preliminary data presented in this chapter suggest that cancer-specific metabolic disruption could be achieved by treating invasive cancer cells with *R. crenulata* extract or one or more of its active ingredients. We have shown that *R. crenulata* affects ROS production and cell viability in a cell-type selective manner. When cells were cultured in anchorage independent growth conditions, which would enrich the progenitor cells population, the change in ROS levels was similar to that of adherent cells, but the viability of the MDA-MB-231 invasive cells decreased significantly only under adherent conditions. This suggests that the effects of *R. crenulata* of invasive cancer cells are influenced by cell-substrate interactions and the genetic make-up of the cells. Our data suggests that alterations in ROS homeostasis by *R. crenulata* in cancer cells could be a possible mediator of the cell-specific effects. However further studies are required to either validate or disprove the role of ROS and identify the molecules/pathways for cancer cells-specific effects of *R. crenulata* extract.
We determined that *R. crenulata* significantly decreased the basal respiration of MDA-MB-231 cells. This is suggestive of reduced mitochondrial metabolism. Our data does not distinguish whether it is a consequence of the respiratory inhibition *versus* substrate limitation to respiratory chain. Increase in ROS could be due to respiratory chain inhibition. However, this alone can exclude the possibility of reduced pyruvate oxidation via the TCA cycle within mitochondria, which provides NADH and FADH$_2$, the respiratory chain substrates. The limitation in pyruvate oxidation may not arise from reduced glucose import and its metabolism by glycolysis as suggested by the higher lactate release from extract treated cells. Therefore, the effect is downstream- at the levels of either pyruvate conversion to Acetyl CoA or pyruvate shuttling towards fermentation. Generally, pyruvate conversion to lactate is favored when pyruvate oxidation is reduced. A respiratory chain inhibition or reduced ATP demand could suppress pyruvate oxidation. Even though the fraction of cellular respiration supporting ATP synthesis is not lower in the extract treated cells, there is increase in glycolysis. This could be due to metabolic re-readjustments in the extract treated cells, which may involve respiratory chain inhibition. Given the identification of a Complex I subunit as target of the metabolites in Rhodiola extracts, it is very likely that Complex I function may be impaired by treatments. This will reduced cellular respiration and result in energy depletion, which might trigger energy-replenishment responses such as autophagy. Our observations support the connection between energy-forming pathways and anti-cancer properties of *R. crenulata* extract. Whether MDA-MB-231 cells indeed enter an energetic collapse that could trigger autophagy will require further analyses, which are beyond the scope of this study. Follow-up studies may provide insight into the mechanisms by which treatment with *R. crenulata* could be selectively detrimental to malignant and not normal cells.
We conclude that metabolic alterations on MDA-MB-231 cells contribute to the observed anti-cancer activity of *R. crenulata*. Furthermore, it is plausible that the overall metabolic effect may result in or be a result of autophagy. Because the extract is a mixture of different ratios of multiple molecules, it is possible such molecules potentially play synergistic roles and that such synergy underlies the anti-cancer activity of *R. crenulata*. The information provided by this study may serve as a foundation for further studies to determine the therapeutic potential of *R. crenulata* bioactive compounds in the treatment of, not only of cancer, but of multiple metabolic diseases.
Figure 22. Effects of *R. crenulata* extract on Reactive Oxygen Species (ROS) levels under different conditions. Cells were seeded in 96 well plates at a density of 1x10^5 cells/well and cultured in phenol red-free medium. Cells were stained with 5μM ROS stain (carboxy-H_2DCFDA). Cells were then treated with 0.05% ethanol (EtOH; orange) or 50μg/mL *R. crenulata* extract (pink) or left untreated (green). The relative fluorescence intensity of H_2DCFDA for each well was measured immediately after treatment addition and every 30 minutes thereafter for a total of 2 hours. A final measurement was performed at 19 hours post-treatment. Cells were incubated at 37°C, 5% CO_2 between measurements. (A) ROS levels in 76NTert-pSUPER non-cancer mammary epithelial cells, (C) 76NTert-siSFRP invasive cells, and (E) MDA-MB-231 invasive breast cancer cells under normal attachment conditions. (B) ROS levels in 76NTert-pSUPER non-cancer mammary epithelial cells, (D) 76NTert-siSFRP invasive cells, and (F) MDA-MB-231 invasive breast cancer cells under ultra-low attachment conditions (similar to AIG).
Figure 23. *R. crenulata* extract decreases 76NTert-siSFRP1 and MDA-MB-231 cell survival under different conditions. 76NTert-siSFRP1 cells were seeded in 96 well plates at a density of 1x10^5 cells/well and cultured in phenol red-free medium. Cells were treated with 2µM Calcein AM. Cells were then treated with 0.05% ethanol (EtOH; orange) or 50µg/mL *R. crenulata* extract (pink) or left untreated (green). The relative fluorescence intensity of Calcein-AM for each well was measured immediately after treatment addition and every 30 minutes thereafter for a total of 2 hours. Live cell assay of (A) 76NTert-pSUPER non-cancer mammary epithelial cells, (C) 76NTert-siSFRP1 invasive cells, and (E) MDA-MB-231 invasive cancer cells under normal attachment conditions and (B, D, F) AIG-like conditions.
OCR vs TIME (Avg)

OCR vs TIME (% of Baseline) (Avg)

On rate (OCR) measurements of MDA-MB-231 reveal that cells treated with R. crenulata have lower basal respiration. Cells pre-treated with 0.05% ethanol (EtOH; blue), 50 μg/mL R. crenulata (yellow) display or left untreated (red) for two hours, and were then treated, sequentially, with oligomycin, FCCP, rotenone, and antimycin A. Data analysis was performed using the Seahorse XF Analyzer standalone Excel-based software and Excel software. The OCR (A) and normalized (by cell equivalents) OCR (B) in each assay group of the three treatment groups after each stress test.
Normalized ECAR of MDA-MB-231 cells treated with 0.05% ethanol or 50 μg/mL *R. crenulata*, and the untreated control. Cells (EtOH; blue), 50 μg/mL *R. crenulata* (yellow) display or left untreated (red) for two hours. Cells were then transferred into XF metabolic stress tests. Details of the experimental procedure can be found in the Materials and Methods section. Measurements m. Cells were then treated, sequentially, with oligomycin, FCCP, rotenone, and antimycin A. Data collected by the Seahorse XF (units) were examined. Each treatment group was assayed in 4–5 replicas, and the full experiment was performed at least twice. Using the Seahorse XF Analyzer standalone Excel-based software and Excel software. The graphs show the average ECAR (A) and free treatment groups after each stress test. The data in (A) was normalized by the protein content (cell equivalents) in each assay to indicate no treatment, vehicle (0.05% ethanol) control, and 50 μg/mL *R. crenulata*, respectively.
## Similarity Ensemble Approach (SEA) Identified Potential Targets for the *R. crenulata* L. (Hook & Thomson, Ohba) compounds identified in this study

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Table 3. Similarity Ensemble Approach (SEA) analysis of FRHB1 series compounds’ topology revealed significant similarity scores between the isolated compounds and known protein ligands, suggesting that the compounds analyzed may bind to the same group of proteins. (Keiser, et al., 2007)
Figure 26. Invasive cells treated with *R. crenulata* display p62 foci near the nucleus that associated with autophagy. (A) 76NTert-pSUPER control cells were treated with 0.1% ethanol (A and B) or 100 µg/mL *R. crenulata* (C and D) under regular (serum-containing) culture conditions. (B) Invasive 76NTert-siSFRP1 cells were treated with 0.1% ethanol (E and F) or 100 µg/mL *R. crenulata* (G and H) under serum-containing culture conditions. p62 foci near the nuclei were taken as evidence of autophagy (Fusco et al., 2012). All treatments lasted 24 hours. White arrowheads indicate p62 foci.
References


cytoskeleton and inhibits HIF-1. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov’t]. Cell Cycle, 7(15), 2409-2417.


ABSTRACT

The regulation of cell polarity is critical for the development and function of all tissues in the body. It is also an important factor in the pathogenesis of cancer, as cancer cells often exhibit aberrant cell polarity. This study aimed to investigate the role of the Par complex in cancer and its relationship with normal cell polarity. The Par complex is a multi-protein complex that plays a key role in regulating cell polarity. In this study, we investigated the expression and function of the Par complex in different cell types and cancer cells.

Results

Our results showed that the expression of the Par complex was significantly lower in cancer cells compared to normal cells. This was accompanied by a reduction in cell polarity, as evidenced by the disorganization of the actin cytoskeleton. Further experiments revealed that the loss of Par complex function was associated with increased migration and invasion of cancer cells.

Conclusions

These findings suggest that the Par complex plays a crucial role in maintaining normal cell polarity and that its dysregulation contributes to the development of cancer. Future studies are needed to further elucidate the molecular mechanisms underlying these processes and to develop new strategies for the treatment of cancer.

BIBLIOGRAPHY


publication of the American Association of Anatomists, 203(2), 163–73. doi:10.1002/aja.1002030205


Lyden, D., Young, a Z., Zagzag, D., Yan, W., Gerald, W., O’Reilly, R., Bader, B. L., et al. (1999a). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature, 401(6754), 670–7. doi:10.1038/44334

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Nieuwkoop, Pieter D. (Pieter Dirk) & Faber, Jacob (1967). Normal table of Xenopus laevis (Daudin): A systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Hubrecht-Laboratorium (Embryologisch Instituut)


Wang, J., Rong, X., Li, W., Yang, Y., Yamahara, J., & Li, Y. (2012). Rhodiola crenulata root ameliorates derangements of glucose and lipid metabolism in a rat model of
the metabolic syndrome and type 2 diabetes. [Research Support, Non-U.S. Gov't]. J Ethnopharmacol, 142(3), 782-788. doi: 10.1016/j.jep.2012.05.063


