Anti-Metastatic and Anti-Tumor growth effects of carnosol on breast cancer

Halima Ali Mohammed Salem Al Samri

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United Arab Emirates University

College of Science

Department of Biology

ANTI-METASTATIC AND ANTI-TUMOR GROWTH EFFECTS OF CARNOSOL ON BREAST CANCER

Halima Ali Mohammed Salem Al Samri

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Rabah Iratni

April 2016
Declaration of Original Work

I, Halima Ali Mohammed Salem Al Samri, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “Anti-metastatic and Anti-tumor Growth Effects of Carnosol in Breast Cancer”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Rabah Iratni, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Our lab has previously showed that carnosol, a naturally occurring polyphenol, exhibits anti-cancer activity by promoting cell cycle arrest at G2 phase, induces reactive oxygen species (ROS) dependent apoptosis and beclin-1 independent autophagy in triple negative breast cancer (TNBC) cell line (MDA-MB-231). Here, we extended our study by investigating the potential effect of carnosol on migration, invasion and tumor growth of these cells. Our findings demonstrated that carnosol, at non-cytotoxic concentrations, exerted a potent anti-metastatic and anti-tumor growth activity against the highly invasive TNBC cell line, MBA-MB-231. Carnosol inhibited the migration and invasion of MDA-MB-231 cells. Moreover, carnosol suppressed the expression and activity of MMP-2 and MMP-9. Remarkably, our investigation revealed that TNF-α and STAT3 proteins that are involved in invasion and metastasis signaling were inhibited in MDA-MB-231 cells in response to carnosol. Furthermore, we demonstrated that carnosol significantly inhibited tumor growth and metastasis in vivo. In conclusion, our findings identify carnosol as a promising chemopreventive and chemotherapeutic candidate that modulate breast cancer growth and metastasis. Hence, carnosol deserves further study and exploration to identify its downstream mechanism(s) of action.

Keywords: TNBC, tumor anti-migration, tumor anti-invasion and tumor anti-metastasis.
تأثير مضادات الانتشار و مضادات النمو في الكارنوسول على سرطان الثدي

الملخص

توصى مخبرنا مؤخرًا أن مركب الكارنوسول (carnosol) وهو مركب طبيعي
يحتوي على مضادات للسرطانة من خلال إيقاف دورة الخلية على مرحلة معينة وانتحار الخلية
من خلال تفعيل صنع المشتقات النافعة للأكسجنيات والالتهاب الذاتي استخدامًا على بيكيلين 
MDA-)( triple-negative breast cancer (beclin-1) في سرطان الثدي ثلاثي السلبية
(MB-231). حالياً، في هذه الطرودة مедьنا دراستنا للتحقق من مضادات السرطنة في
الكارنوسول على هجرة واجتياح ونمو الثدي السرطانية. ونوصي مؤخرًا بأن الكارنوسول على
تراكيز غير سامة قادر على منع انتشار ونمو هذه الخلايا السرطانية وهي خلايا سرطان الثدي
ثلاثي السلبية. في الواقع، فقد قررنا أن الكارنوسول قادر
على منع هجرة وعمر ونحو خلايا سرطان الثدي (MDA-231). وإضافة إلى ذلك وجدننا أن
الكارنوسول قادر على تثبيت تعويض ونشاط هذه البروتينات (MMP-2 and MM-9).
وقد نظرنا أيضاً مقارنة ثبتية الكارنوسول على منع هذه البروتينات (TNF-α and STAT3)
التي تتضمن في مسار هجرة واجتياح هذه الخلايا. وعلاوة على ذلك، أثبتنا أهمية الكارنوسول على
منع نمو وانتشار السرطان على الكائن الحي (in vivo). وفي الختام، النتائج التي توصلنا إليها
ظهرت الكارنوسول على أنه معالج كيميائي وواقي (chemopreventive and
(chemotherapeutic)
وبالتالي، الكارنوسول يستحق المزيد من الدراسة والاستكشاف لتحديد آلية العمل.

مفاهيم البحث الرئيسية: سرطان الثدي ثلاثي السلبية، مضاد الانتشار، مضاد اجتياح، مضاد
نمو السرطان.
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Though only my name appears on the cover of this thesis, yet many people have contributed and were involved in its production. I owe my gratitude to all those people who helped and supported me directly or indirectly throughout my years of study and to whom my graduate experience has been one that I will cherish forever.

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Dedication

To my beloved mother (Khadija Hamda Al Dhaheri), for her prayers to me and continuous support, my father’s soul (Ali Mohammed Al Samri), the first to teach me and who I miss every day, and my family for care and support all the time
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<th>Description</th>
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<tbody>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>Ki67</td>
<td>Proliferation Marker</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple Negative Breast Cancer</td>
</tr>
<tr>
<td>CAMs</td>
<td>Cell Adhesion Molecules</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>BM</td>
<td>Basement Membrane</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix Metalloproteinases</td>
</tr>
<tr>
<td>EMMPRIN</td>
<td>Extracellular Matrix Metalloproteinase Inducer</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia Inducible Factor-1</td>
</tr>
<tr>
<td>PlGF</td>
<td>Placental Growth Factor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal- Epithelial Transition</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin Gallate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>pERK1/2</td>
<td>Phosphorylated Extracellular Signal-Regulated Kinase 1 and 2</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Cancer

1.1.1 Brief history of cancer

Cancer has been known to mankind since ancient times. Cancer initiates when cells in a certain part of the body start to grow out of control. The study of cancer is termed oncology. One of the first evidence of the existence of cancer was discovered in fossilized bone tumors in human mummies in ancient Egypt. As it was referenced in ancient manuscripts, bony skull damage was seen in the head and neck also. Although the term cancer was not used, the oldest explanation of cancer was found in a book called “Edwin Smith Papyrus” which dates back to about 3000 BC[1]. The word cancer was coined by the Greek physician, Hippocrates who is considered as the father of medicine (460-370 BC). Hippocrates used the Greek terms, carcinos and carcinoma to describe tumors. In fact, this term is used to describe a crab movement, which he thought behaved like a tumor[2], [3].

1.1.2 Cancer epidemiology

Cancer is a leading cause of death in both developed and developing countries. Recently, cancer diseases have become a global health problem. The burden of cancer incidence is expected to increase worldwide from 14.1 million cases in 2012 to 21.6 million by 2030. In 2012, 8 million cancer related deaths were recorded worldwide[4]. One of the main reasons for the rising burden of cancer is the aging of the population. Furthermore, tobacco and alcohol consumption, obesity alongside other risk factors lead to an increase in cancer occurrence[5], [6].
1.1.3 Cancer characteristics

Cancer is known as a class of disorders or diseases characterized by uncontrolled cell growth and/or defects in vital and critical control mechanisms such as the ability to undergo cell death or growth arrest. Additionally, cancer cells are characterized by their capability to spread from the primary site, or site of origin, to adjacent tissue through invasion, or by embedding into distant sites by metastasis. Moreover, cancer cells acquire autonomous growth signals, escape from growth inhibitory signals, enable replicative immortality, induce angiogenesis (formation of new blood vessels)[7], [8], genome instability and tumor-promoting inflammation which are crucial characteristics of cancer. Additionally, they highlighted two other merging hallmarks that required further study which are avoiding immune destruction and reprogramming energy metabolism[9].

1.1.4 Classification of cancer

Cancers are very diverse, requiring different treatments and diagnoses. More than one hundred different types of cancers have been identified. Cancers can be classified according to the type of tissue in which they originate. There are five main groups, which are carcinoma, sarcoma, myeloma, leukemia and lymphoma. Carcinoma start in epithelial tissues, while sarcoma is found in mesoderm derived cells (such as bone and muscle) and myeloma originate in the plasma cells of bone marrow. Moreover, leukemia is a cancer that is found in the bone marrow and lymphoma develops in the glands or nodes of the lymphatic system[8], [10].
1.2 Breast cancer

It is noteworthy to mention that breast cancer is one of the major causes of death among women throughout the world. Breast cancer is a malignant tumor that originates from breast cells. A malignant tumor is made up of a group of cancer cells that can invade nearby tissues or metastasize to distant areas of the body. Breast cancer occur mostly in women, although men can have it, too[11].

Understanding the normal breast structure is necessary in order to understand the breast cancer disease. Breast tissues are made up mainly of lobules (glands that produce milk), ducts (tubes that transport the milk from the lobules to the nipple and it has contracting function) and stroma (connective and fatty tissues surrounding the lobules and ducts, blood vessels, and lymphatic vessels)[10], [12], [13]. Cancers initiating from ducts are known as ductal carcinomas; while those starting from lobules are known as lobular carcinomas[14], [15]. There are a number of factors correlated with an increased risk of breast cancer such as age, family history, exposure to radiation[41] and lifestyle[16].

1.2.1 Descriptive epidemiology

Breast cancer is a serious disease and by far the most frequently diagnosed cancer in women on a global scale. In 2012, there were 1.7 million cases and 0.5 million breast cancer deaths. The incidence of breast cancer accounted for 25.2% of all cancer cases and 15% of all cancer deaths in women in 2012. Moreover, breast cancer incidence rates in developing countries was 2-fold higher than developed countries and the death rates were found to be 2 to 5-fold higher in developing
countries than in developed countries[5]. It is predicted that by 2030, the number of new cases of cancer in the world will increase to 22.2 million[17], [18].

In the United Arab Emirates (UAE), cancer is considered to be the third leading cause of death after heart diseases and accidents, where cancers cause 15% of total deaths. In 2012, 20% of diagnosed cancer cases was breast cancer. Moreover, breast cancer is considered to be the leading cause of death among other cancers like lung and colon cancers, where it represents 13.3% of all other cancers[19].

1.2.2 Molecular classification of breast cancers

Breast cancer is considered to be a highly heterogeneous group of cancers that arrive from different cell origins and each has its own clinical implications. Breast cancer is classified according to the histopathology, tumor grade, tumor stage, and protein profile and gene expression[20].

The molecular classification of breast cancer is based on examining the alterations of gene expression that drive cancer. This is important as it has prognostic significances beyond the traditional prognostic indexes and can aid in the determination of the most suitable treatment for the individual. Accordingly, at the molecular level, breast cancer can be categorized into five subtypes which are luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) overexpression, basal-like tumors and normal like tumors[21]–[23]. Normal-like tumors are poorly classified and they need more studies and their clinical significance is still undetermined[24].
1.2.2.1 Luminal tumors

Most breast cancers are luminal-like tumors, representing 64.3% of other breast cancer subtypes. They are highly identified among hormone receptor-positive cancers like estrogen (ER) and progesterone (PR) receptors[25]. There are two subtypes of luminal tumors, i.e., luminal A and luminal B[22]. Luminal A breast cancer has been defined as ER+/HER2- and has low Ki67 (proliferation marker)[26], while luminal B as ER+/HER2- and has high Ki67[27]. Luminal B tumors are more aggressive in comparison with luminal A and have higher histological grade and poorer diagnosis. The treatment of this subtype of breast tumor is mainly based on endocrine therapy[28].

1.2.2.2 HER2 over-expression tumors

HER2 is a cell membrane receptor tyrosine kinase and it is usually involved in the signal transduction pathways leading to cell differentiation and growth[29]. Approximately 15% to 20% of all breast cancers correspond to HER2 over-expression[20]. HER2 is distinguished for its role in the pathogenesis of breast cancer and as a target for therapy. HER2 over-expression tumors are highly proliferative and aggressive. They have a high histological grade, poor prognosis, and are highly sensitive to chemotherapy[30]. Recently, combination of anti-HER2 therapies showed promising results in response rate and tolerance[31], [32].

1.2.2.3 Basal-like tumors

Basal-like breast cancers represents around 10% to 20% of breast carcinomas. The term basal-like was coined because this kind of tumors expresses genes that are usually found in normal breast myoepithelial cells[33]. According to immunohistochemical markers, there are several proposed types of basal-like tumors
and one of them is the triple negative breast cancer (TNBC) which is negative to ER, PR and HER2 receptors[34], [35]. Basal-like breast cancers have different clinical presentations, diverse response to chemotherapy, distinctive histological features and various outcomes[34], [36].

1.2.2.3.1 Triple negative tumors

TNBCs represent 60% to 90% of all basal-like cases and is known to lack the expression of ER, PR and HER2[22]. Triple negative is considered to be highly aggressive and have high proliferative index compared to other breast cancers[37]. Moreover, it is characterized by distinctive molecular profile and patterns of metastasis which usually include brain, lung and bone metastasis[38]. Additionally, they have poor prognosis and relapse very quickly compared to other breast cancer subtypes[39]. Also, some of the TNBCs are known to have BRCA1 mutations. BRCA1 is essential for DNA repair mechanisms and its inactivation accumulates DNA errors and cause genetic instability which could lead to tumor growth[40].

1.3 Targeted pathways and cellular process in breast cancer

At early stages of tumor progression, cancer cells multiply near the site of origin where their ancestors first started their uncontrolled proliferation. This results in a primary tumor where the cancer cells will proliferate and expand within the organ of origin. These primary tumors correspond to about 10% of cancer deaths. However, the remaining 90% of deaths were found to have metastasized tumors[41], [42]. Metastasis action is followed by the formation of new blood vessels in a process known as angiogenesis[8]. Therefore, better understanding of the molecular
mechanisms of metastasis and angiogenesis is important to develop new treatments and prevention strategies against breast cancer.

1.3.1 Metastasis

Metastasis is defined as a cascade of events which are connected through a series of adhesive interaction and invasive processes, along with responses to chemotactic stimuli[43], [44]. Metastasis has been characterized as a late stage in cancer progression. The formation of metastasis cascade is extremely complex where it is composed of several events which correspond to several biological functions. The steps involved in metastatic process are: (1) invasion, (2) intravasation, (3) transport, (4) extravasation and (5) metastatic colonization[8], [45], [46] (Fig. 1).

Figure 1: Schematic diagram showing the different stages of cancer metastasis. Figure modified from[47].
Cancer cells can’t be viewed as an isolated mass of tumor, but instead as tissue that receives and recruits signals from surrounding cells, which is termed tumor-associated stroma or tumor microenvironment. Tumor microenvironment affects the interactions of tumor cells and its ability to metastasize. Breast tumor microenvironment is composed of extracellular matrix and different cell types, such as fibroblasts, immune cells, endothelial cells and adipocytes[48]. All of these components play a role in the carcinogenesis of breast cells and their ability to metastasize.

1.3.1.1 Invasion

Invasive and migratory abilities of cancer cells are considered as key features of metastatic cascade. In order for cells of primary tumor to invade surrounding tissue, they must escape from the normal molecular restrictions that link nearby cells to each other. Thus, these tumors cells need to remodel their cell-matrix and cell-cell adhesion interactions to gain invasive capabilities[8].

Cell adhesion molecules (CAMs) are families of proteins that mediate heterotypic (distinct cell types) and homotypic (same cell types) recognition[8]. Cadherin is one of the CAMs and it is a transmembranal glycoprotein that interact with actin component of the cytoskeleton through catenins[49]. Catenins are involved in the maintenance of tight intracellular adhesion interactions. Cadherin-catenin complexes are also involved in the intracellular signal transduction cascades[50]. For instance, cadherin is known to sequester catenin, preventing catenin from the association with other downstream signaling, thus possibly suppressing oncogenic signals[51]. E-cadherin is a member of the cadherin family
and its abrogation has been shown to be associated with breast carcinoma and showed fibroblast-like and invasive phenotypes[52]–[55]. Moreover, breast cancer was shown to display either down-regulation or complete loss of α-, β- and γ-catenins[50], [56]. However, Hazan et al. (2000) found that transfection of α-catenin gene into metastatic epithelial cells resulted in the restoration of intracellular adhesion[57]. Moreover, another study showed that the transfection with E-cadherin cDNA resulted in the re-establishment of epitheloid phenotype and abrogate their invasiveness.

Tumor cells must also break free from the interaction with the normal extracellular matrix (ECM) components. The interaction between epithelial cell and ECM involves integrin receptors. Integrin receptors bind to basement membrane (BM) and ECM through the recognition and interaction with various components of ECM, such as collagen, fibronectin or laminin, depending on the subunits that form the integrin[8], [58]. However, altered expression of integrin is found to be involved in breast carcinomas and to facilitate its invasion. Throughout de-adhesion to surrounding ECM, consequently tumor cells will be allowed to escape the primary tumor.

Invasion of cancer cells into adjacent tissues require the action of several hydrolytic enzymes or proteases, which are released either by cells around the tumor or by the tumor cells themselves[59]. Matrix metalloproteinases (MMPs) and serine proteases are two families of proteolytic enzymes implicated in metastasis. The MMPs family include several proteases, such as gelatinases, collagenases, membrane-type MMPs and other proteases[60]. MMPs production is induced via protein named extracellular matrix metalloprotease inducer (EMMPRIN). These
MMPs act on degrading the structural components of ECM and BM and cleave other proteins found outside the cells, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)[61]. Consequently, these proteins (VEGF and FGF) play a crucial role in metastasis and stimulate angiogenesis. Up-regulation of MMPs were found to be associated with breast, lung and colon tumors.

One characteristic of invasive tumor is coordinated by a process termed epithelial-mesenchymal transition (EMT) which comprises the conversion of a sheet of closely connected epithelial cells into highly mobile mesenchymal cells. This process is found commonly at early stages of embryogenesis. Its characterized by low expression of epithelial markers (e.g., E-cadherin) and high expression of mesenchymal proteins (e.g., N-cadherin) and MMPs. These characteristics were observed in metastasized tumors[8], [62]. Currently, several therapeutic agents are under study for their capability to decrease cancer invasiveness based on invasion’s regulators and their inhibitors[63].

1.3.1.2 Intravasation

Intravasation involves the entry of invasive carcinoma cells into lymphatic or blood vessels and transport them to distant tissues[64]. This process involves several steps: (1) cancer cells must adhere to vessel wall, (2) basement membrane destruction via MMPs, and (3) transendothelial migration where carcinomas cell pass between the endothelial cells into the bloodstream[8], [65].
1.3.1.3 Transport

Transport and circulation of carcinoma cells through the bloodstream and lymphatic vessels is in one direction[8]. Once tumor cells enter the bloodstream, they are exposed to shear forces and interactions that might lead to their destruction. Though, cancer cells are capable of resisting such destruction by attaching to the endothelia cells of blood vessels and thereby protecting themselves from the immunesystem[66].

1.3.1.4 Extravasation

Extravasation is the escape of tumor cells from lymphatic or blood vessels to initiate secondary metastatic out-growth, and it can be taken as the reverse of invasion[59], [67]. This process involves the adherence of tumor cells to endothelial part of the blood vessel, penetration of endothelial wall, passage through the basal membrane, and migration into surrounding stroma[8], [10].

1.3.1.5 Metastatic colonization

Metastatic colonization is the formation of a progressively growing tumor at the secondary tissue. The microenvironment of the new metastatic site differs completely from the site of primary tumor. Cancer metastasis is an organ-selective event, where carcinomas cells will spread to specific and suitable environment in which they grow and develop[68].

1.3.2 Angiogenesis

Successful tumor metastasis cannot be completed without angiogenesis. Angiogenesis is the process of forming new blood vessels. This process has an
essential role in the formation of new vascular network to provide oxygen and nutrients and to remove waste products from cancer cells[69]. Recently, the involvement of angiogenesis in tumor progression has been extensively studied.

1.3.2.1 The process of angiogenesis

The regulation of angiogenesis depends on a net balance between anti-angiogenic factors and pro-angiogenic factors. Pro-angiogenic factors involve VEGF, tumor necrosis factor (TNF-α) and other factors that are secreted from tumor cells or mobilized from the ECM. The first event in angiogenesis is the ‘angiogenic switch’ which is the switch of non-angiogenic tumor to be angiogenic tumor[8]. The angiogenic switch in tumors involves two main events: (1) up-regulation of pro-angiogenic factors and/or down-regulation of anti-angiogenic factors; (2) hypoxia that activates hypoxia inducible factor-1 (HIF-1), resulting in VEGF production[70].

The process of angiogenesis involves (1) the production of cytokines (by carcinoma and surrounding stromal cells) and proteases (by endothelial cells) to degrade vascular BM, (2) the migration and proliferation of endothelial cells through the breached BM towards tumor cells and formation of endothelial tube (3) recruitment and attachment of mesenchymal cells to the endothelial cell tube and blood vessels maturation with the formation of vascular basement membrane[64], [71]. Several ECM proteins and angiogenic factors are involved in angiogenesis.

Upon stimulation of angiogenic factors, proteases such as MMPs get activated and start degrading vascular BMs leading to the migration of activated endothelial cells into interstitium. Proteases also fragment some ECM proteins such as type IV collagen, which leads to the migration of endothelial cells[72].
Additionally, some of the matrix components, such as fibronectin, fibrin, and others were found to guide endothelial cells to their targets and provides a supportive scaffold, and subsequently facilitating the formation of neovessels[73].

1.3.2.2 Factors involved in regulating angiogenesis

1.3.2.2.1 Angiogenic inducers

Several growth factors and endothelial-specific factors (i.e., VEGF) are involved in the induction of angiogenesis. The role of VEGF in angiogenesis regulation has been intensely investigated. VEGF is one of the first player in the initiation process of angiogenesis. VEGF-signaling is vitally involved in physiological and pathological angiogenesis specifically in tumor growth. The VEGF gene family comprises of VEGF-A, VEGF-B, VEGF-C, and placental growth factor (PlGF)[74]. VEGF-A is a key regulator of blood vessel growth, and VEGF-C and VEGF-D are required in lymphatic angiogenesis regulation[8].

VEGF induces the proliferation and growth of endothelial cells in vitro, and provokes angiogenic response in vivo[74], [75]. Additionally, it induces the survival of endothelial cells through activating phosphatidylinositol (PI)-3 kinase-Akt pathway and producing anti-apoptotic proteins[76], [77]. Moreover, it promotes vascular permeability, causing inflammation and other pathological conditions[78], [79]. Also, over expression of VEGF has been studied in various human malignancies including breast, colon, and lung cancers. An in situ hybridization study of human breast carcinomas samples showed high VEGF expression, compared to normal breast cells[80]. Subsequently, VEGF might be a good target for breast cancer treatment.
1.4 Breast cancer treatments

Today’s therapies for breast cancer include several approaches consisting mainly of surgery, radiation therapy, chemotherapy, hormonal therapy and combined therapy. These approaches can all be used in both the curative and the maintenance of curation from breast cancer.

1.4.1 Conventional treatments for breast cancer

Once breast cancer gets diagnosed, usually the treatment is started with either surgical removal of discrete tumor from breast (lumpectomy) or the entire diseased breast (mastectomy) following adjuvant systemic therapy to decrease the risk of relapse. Mastectomy is found to be effective therapeutic approach for breast cancers with BRCA mutations[81]. Also, it is commonly used for invasive breast cancers at early stages[82]. Lumpectomy is used alone or in combination with radiotherapy, and it is reported to be more suitable therapy for invasive breast cancer[83].

Radiotherapy is the exposure to a specific ionizing radiation from an external source which will ultimately damage the cells through direct ionization of DNA, mediated by (reactive oxygen species) ROS generation via radiation-induced water hydrolysis and production other toxic radicals. Moreover, Radiotherapy disturbs cellular homeostasis which will trigger the apoptosis pathway. Radiotherapy has an important role in the reduction of cancer recurrence and it is applied usually after surgery[84].

Usually chemotherapy is applied immediately after surgery or before surgery[85]. Chemotherapy is recommended for all women with invasive breast
cancer that is greater than 1 centimeter. Neo-adjuvant chemotherapy is used to reduce the size of a tumor and to prevent the development of micro-metastasis before the surgery. Adjuvant chemotherapy is used to reduce the risk of relapse [86]. There are different chemotherapy regimens that are used, each based on specific characteristic of the tumor type and status. Generally, the main effect of chemotherapy is believed to kill cells by inducing apoptosis in the malignant cells by targeting specific cellular mechanisms[87], [88].

Breast cancer develops in epithelial tissues of the breast, which are known to proliferate under hormonal control. Therefore, breast cancer is often treated with endocrine modulators when tumor cells retain their hormonal receptors. For instance, tamoxifen, an ER regulator which binds to ER and blocks estrogen from binding to it, is usually given to premenopausal women. However, endocrine therapy fails to cure TNBCs as they lack hormonal receptors[89].

1.4.2 Targeted treatments for triple negative breast cancer

TNBCs do not respond to hormonal therapy and they are highly resistance, aggressive, metastasize and invasive. At present, many researches are going on to further characterize the TNBCs and find target therapies for them. Some clinical studies suggested new agents that are effective on the triple negative tumors.

1.4.2.1 Metastasis inhibitors

While metastasis is the main cause of death in breast cancer patients, anti-metastatic therapeutic agents are rarely developed. Interestingly, Esak Lee and his group reported a novel peptide (called collagen IV peptide SP2012) which inhibits
the growth and metastasis of breast cancer. SP2012 is found to inhibit breast cancer growth, metastasis, blocks angiogenesis and lymphangiogenesis. This SP2012 peptide inhibits lymphatic and blood endothelial cell viability, migration, adhesion, and tube formation by targeting MET (mesenchymal-epithelial transition) signals[90].

1.4.2.2 Angiogenesis inhibitors

TNBCs are known to be highly aggressive with enhanced angiogenesis and have been found to have high expression of VEGF. A discussed earlier, VEGF is a key mediator of angiogenesis, it stimulates endothelial cell proliferation and regulates vascular permeability in breast cancer[91]. Therefore, targeted therapy against angiogenesis can be preventive for breast cancers.

One agent involves targeting angiogenic factors such as VEGF is bevacizumab. Bevacizumab is a humanized recombinant monoclonal antibody which recognize all known isoforms of VEGF[92]. A study showed that bevacizumab alone has no effect on breast cancer in terms of tumor growth delay. However, a combination of bevacizumab with chemotherapy (i.e., docetaxel) showed 47% reduction in tumor progression[93].

1.4.3 Plant-derived anti-cancer agents

Natural plants have been used as medicine to prevent and to cure several diseases for thousands of years. Plants have been found to be an excellent source of bioactive compounds which have beneficial health effects, and very often, these plants are edible and used in culinary. Certain bioactive compounds from plants have
been found to have anti-cancer activities, therefore, plants became one of the main interests for researchers.

1.4.3.1 Phytochemicals

Phytochemicals are natural compounds that are derived from plants. Thousands of phytochemicals have proved to possess chemo-preventing activities against cancers without having severe side-effects compared to conventional treatments[94]. Many cancer therapy researches have aimed to identify and develop new chemotherapeutic agents that are derived from plants. Different phytochemicals found in diet were tested and were found to have toxic effects on breast cancer cells \textit{in vitro} and prevent breast cancer progression \textit{in vivo}[95]. Moreover, phytochemicals have been shown to target breast cancer through inhibiting cellular proliferation, suppressing inflammatory response, arresting cell cycle, causing apoptosis and modulating gene expression (such as up-regulation of tumor suppressing genes and suppressing angiogenic and invasion genes)[96].

Examples of plant-derived agents in preclinical development are paclitaxel and docetaxel taxanes. These are compounds obtained from yew tree and they showed anti-tumor activity against metastatic breast cancer and ovarian cancer. They also induced mitotic arrest and apoptosis[97], [98]. Another example is DNA topoisomerase I inhibitor β-lapachone obtained from the Lapacho tree. It induces cell cycle arrest at G1 or S phase and causes apoptotic or necrotic cell death in different type of human carcinoma cells, including breast, ovaries, colon, lungs and prostate[99]–[101]. Additionally, apigenin is a flavone compound extracted from vegetables such as chamomile, celery and other plants. It exerts cytotoxic activities
against breast cancer and colon cancer cell lines[102]. Apigenin also has a chemoprevention activities against cancer cells and induces a process of autophagic cell death[103]. Another plant-derived agent in clinical was derived from a spice *Bleekeria vitensis*. Elliptinium was found to have anti-breast cancer activitiy and is marketed in France nowadays[104].

During the past decades, there has been substantial progress in identifying how certain phytochemicals are able to interfere with specific events in the process of carcinogenesis. Epigallocatechin gallate (EGCG) is a chemopreventive and anti-oxidant polyphenol that is found in green tea. It has been shown that EGCG suppressed VEGF production via inhibiting the activation of both STAT3 and NF-κB in human breast cancer cell lines[105], [106]. NF-κB has been reveled to be anti-apoptotic and it stimulates tumor proliferation, while STAT3 is found to be associated with tumor metastasis and angiogenesis[107]. Further investigation, are needed to understand the anti-carcinogenic actions of phytochemicals.

### 1.4.3.1 Carnosol

Since prehistoric times, mediterranean herbs such as sage and rosemary have been used for their medicinal properties and culinary purposes. Sage and rosemary have been known to contain various polyphenolic compounds such as carnosol, rosmanol, carnosic acid, rosmarinic acid as well as others[108]. Carnosol was first isolated from sage in 1942 and its chemical structure identified by Brieskorn *et al.* in 1964[109]. Carnosol is a phenolic diterpene and is synthesized from carnosic acid[110], [111]. Carnosol was reported to have potent anti-oxidant, anti-inflammatory and anti-cancer properties[112].
The accumulation of ROS and the reduction of anti-oxidants have been known to promote various biological responses including inflammatory conditions, neurodegenerative disease, cardiovascular disease, and carcinogenesis of several tissues[113]. Rosemary extracts were revealed to have radical scavenging activity which was mainly attributed to carnosol and carnosic acid[114]. Moreover, deregulated inflammatory signaling lead to excess nitric oxide (NO) production that occur during inflammation and the multi-step process of carcinogenesis. This encouraged the search for agents that decrease inflammatory signaling cascades. Carnosol was reported to reduce nitric oxide production through the inhibition of NF-kB, p38 and mitogen activated protein kinase (MAPK) in mouse macrophages which provide possible mechanisms for anti-inflammatory action[115].

In addition, carnosol has been shown to elicit chemopreventive effects by blocking the activation of carcinogens, inhibiting cell proliferation, inducing apoptosis selectively in cancer cells, and blocking tumor angiogenesis and invasion[116]. Carnosol repressed proliferation and stimulated apoptosis in several human cancer cells. This compound was found to induce cell cycle arrest at G2/M phase and to decreased mitotic exit of human colon cancer (Caco-2) cells through up-regulation of cyclin B expression[117]. Similarly, carnosol induced G2/M phase cell cycle arrest in human prostate cancer (PC3) cells through down-regulation of several cyclins (A, D1, and D2), and cyclin-dependent kinases (Cdk), and up-regulation of Cdk inhibitors (i.e., p21 and p27). Furthermore, carnosol caused apoptosis cell death in PC3 cells via activation of caspase-8 and -9, stimulation of Bax and inhibition of B-cell lymphoma-2 (Bcl-2) expression[118]. The anti-proliferative activity of
carnosol in human leukemia (HL-60) cells were associated with G1 arrest, caspase-3 activation and induction of apoptosis[119].

Moreover, carnosol exhibits anti-angiogenic and anti-metastatic properties. This phenolic compound was found to prevent the migration of human umbilical vein endothelial cells (HUVEC) and human bronchial aortic endothelial cells (BAEC) and decreased activity of MMP-2[119]. Also, carnosol repressed the migration of metastatic mouse melanoma (B16/F10) cells in vitro by inhibiting the expression of MMP-9[120], [121]. In vivo studies, were survival of rats with acute myeloid leukemia was improved by administration of carnosol alone or in combination with vitamin D, which revealed a strong anti-proliferative effect[122], [123]. Additionally, it was able to decrease tumor multiplicity in a mouse model with colon cancer[124].

1.4.3.1.1 Carnosol and breast cancer

Carnosol was investigated for its anti-breast cancer properties. This compound has been shown to have cytotoxic activity against MCF-7 human breast cancer cells[125]. Likewise, carnosol was observed to decrease the cellular viability of T47D[126], AU565[127], and MDA-MB-231 human breast cancer cells[128]. Additionally, the anti-growth activity of carnosol as a single agent or in combination with other chemotherapeutic drugs or phytochemicals against breast cancer cell line was evaluated. They found that carnosol reduced cellular viability in human breast cancer, and suppressed the adhesion of cancer cells to fibronectin. The combination therapy with other phytochemicals such as curcumin increased the anti-proliferative activity of carnosol and resulted in a synergistic reduction of viability in MDA-MB-
231 cells. These results provide additional evidence about the anti-breast cancer role of carnosol and its potential activity in blocking the growth of tumor cells[129].

Recently, our lab investigated the *in vitro* and *in vivo* anti-cancer effects of carnosol in TNBC and the data were consistent with a model shown in Figure 2[128]. They found that carnosol significantly inhibited cellular viability and colony growth and induced G2 arrest in the MDA-MB-231. Cell cycle blockade was associated with up-regulation of p21/WAF1 expression and down-regulation of p27. Furthermore, carnosol was found to induce beclin1-independent autophagy and apoptosis in MDA-MB-231 cells. The coexistence of both events, apoptosis and autophagy, was confirmed by electron microscopy. Autophagy induction was found to be an early event, detected within 3 hours post-treatment, which consequently led to apoptosis. Carnosol treatment also caused an increase in the levels of phosphorylated extracellular signal-regulated kinase 1 and 2 (pERK1/2) in a dose-dependent manner. Additionally, they showed that carnosol induced DNA damage, reduced the mitochondrial potential and prompted the activation of the intrinsic and extrinsic apoptotic pathways. Moreover, they found that carnosol induced a dose-dependent production of ROS, and inhibition of ROS by tiron (a ROS scavenger), prevented the induction of autophagy and apoptosis and attenuated DNA damage. These findings provide a strong evidence that carnosol may be an alternative therapeutic agent against the aggressive form of breast cancer and thus deserves more exploration[130]. Here, therefore, we decided to expand our study by investigating the potential effect of carnosol on cell migration, invasion and tumor growth of the TNBC.
Figure 2: Hypothetical model demonstrating the differential effect of carnosol in MDA-MB-231 triple negative breast cancer cells[128].
Chapter 2: Materials and Methods

2.1 Cells culture and reagents

Human breast cancer cells MDA-MB-231 were maintained and cultured in Dulbecco Minimal Essential Medium (DMEM) (Hyclone, Cramlington, UK). The media was complemented with 10% fetal bovine serum (FBS) (Hyclone, Cramlington, UK), 100 U/ml penicillin/streptomycin (Hyclone, Cramlington, UK). Carnosol was obtained from Sigma Aldrich. TNF-α antibody was obtained from Abcam. Antibodies to pSTAT3, β-actin, goat anti-mouse IgG-HRP (sc-2005) and goat anti-rabbit IgG-HRP (sc-2004) were obtained from Santa Cruz Biotechnology, Inc. LY294002 was purchased from Sigma-Aldrich.

2.2 Wound healing migration assay

In brief, MDA-MB-231 cells were grown in 6-well tissue culture dishes until they reach confluence stage. A scrape was made over the confluent monolayer with a sterile plastic pipette tip of 1 mm diameter. Three wounds were made for each sample or well. Afterwards, the plates were washed twice with 1X PBS and incubated at 37°C in fresh DMEM supplemented with 10% fetal bovine serum in the presence of vehicle (Dimethyl Sulfoxide (DMSO)) or indicated concentrations of carnosol. At the bottom side of each dish, three random places were marked where the width of the wound was photographed with an inverted microscope at 40X magnification (Nikon Ti-U, Nikon). Wound closure was determined by measuring the distance (µm) between the edges of the wound at time 0, 6 and 24 hours, using the NIS-Elements BR 3.0 software (Nikon). Quantification of the distance migrated
by the cells was done as follow: \( D = (\text{Size of the wound at } t = 0 \text{ h} - \text{size of the wound at } t = 6 \text{ or } 24 \text{ hours}). \)

2.3 Matrigel invasion assay

Invasiveness of the MDA-MB-231 cells treated with carnosol was tested using BD Matrigel Invasion Chamber (8-µm pore size; BD Biosciences, Bedford, MA, USA) according to manufacturer’s instructions. Briefly, MDA-MB-231 cells (1 \( \times 10^5 \)/well) were placed in 0.5 mL of media containing vehicle (0.2% DMSO) or the indicated concentration of carnosol were seeded into the upper chambers of the system; the bottom wells in the system were filled with DMEM complemented with 10% fetal bovine serum as a chemo-attractant and then incubated at 37°C for 24 hours. Non-penetrating cells were removed from the upper surface of the filter with a cotton swab. Cells that have migrated though the Matrigel were fixed with 4% formaldehyde, stained with DAPI. Fluorescence from DAPI was detected using a filter with excitation wavelength of 330–380 nm and barrier filter of 400 nm. DAPI-stained nuclei were counted in 10 random fields per well using inverted fluorescence microscope at 200X magnification (Nikon Ti-U, Nikon). For quantification, the assay was done in duplicate and repeated three times.

2.4 Measurement of matrix metalloproteinases by ELISA

Cells were seeded in 6-well tissue culture plates in the presence of vehicle (DMSO) or carnosol for 24 hours. The conditioned medium was collected and the levels of secreted MMP-9 and MMP-2 were measured using immunoassay kits (Invitrogen, Camarillo, CA, USA) according to the manufacturer’s protocol.
Experiments were repeated three times and the average of three means is represented ± SEM.

2.5 Gelatin zymography

Gelatin zymography was performed according to Kleiner and Stetlerstevenson’s protocol[131]. Briefly, MDA-MB-231 (2.5 x 10^6) cells were incubated in serum-free medium for 24 hours in the presence of vehicle (DMSO) or carnosol (25 µM and 50 µM). The conditioned medium was collected, concentrated and 30 µg of total protein was determined in 10% polyacrylamide gels containing 0.1% gelatin. Following electrophoresis, the gels were washed for 1 hour in 2.5% (v/v) Triton X-100 to remove SDS and then incubated overnight at 37°C in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM ZnCl_2 and 10 mM CaCl_2 to allow proteolysis of the gelatin substrate. Bands corresponding to protein activity were visualized through negative staining using 0.5% Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA, USA). Illustrative results from two independent experiments are shown.

2.6 RNA extraction and RT-PCR

Total RNA from vehicle- or carnosol-treated MDA-MB-231 cells were prepared using Trizol reagent as described by the manufacturer (Life Technologies, Inc. Grand Island, NY, USA). RNA expression of MMP-9 was determined by RT-PCR. RT-PCR was performed using the Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany) according to manufacturer’s instruction. Equal amounts of RNA (500 ng) were used as templates in each reaction. The sequences of specific primers were as follows: MMP-9 sense, 5’-TTGACAGCGACAAGAAGTGG-3’, and
antisense, 5′-CCCTCAGTGAGCGGCATC-3′; GAPDH sense, 5′-GAGCTCCAAGGAGTAAGACC-3′, and antisense: 5′-AGGGGTCTACATGGCAACTG-3′. The PCR products were separated by 1.5% agarose gel and visualized by ethidium bromide staining. Representative results from two independent experiments are shown.

2.7 Western blotting

Cells (1.8 x 10⁶) were seeded in 100 mm culture dishes and cultured for 24 hours before addition of carnosol at the indicated concentrations. After incubation, cells were washed twice with ice-cold PBS, scraped, pelleted and lysed in RIPA buffer (Pierce) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche). After incubation for 30 min on ice, cell lysates were centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentration of lysates was determined by BCA protein assay kit (Thermo Scientific) and the lysates were adjusted with lysis buffer. Aliquots of 25 μg of total cell lysate were resolved onto 8–15% SDS-PAGE along with PageRuler Plus Prestained Protein Ladder (Thermo Scientific). Proteins were transferred to nitrocellulose membranes (Thermo Scientific) and blocked for 1 h at room temperature with 5% non-fat dry milk in TBST (TBS and 0.05% Tween 20). Incubation with specific primary antibodies was performed in blocking buffer overnight at 4°C. Horseradish peroxidase-conjugated anti-IgG was used as secondary antibody. Immunoreactive bands were detected by ECL chemiluminescent substrate (Thermo Scientific). Where needed, membranes were stripped in Restore western blot stripping buffer (Thermo Scientific) according to the manufacturer’s instructions.
2.8 Animal studies

All animal experiments were conducted in accordance with the French guidelines on the use of animals in scientific investigations with the approval of the local Ethical committee, (Institut Albert Bonniot, Grenoble, France: licence protocol N°381029 and B3851610001).

2.9 Chick embryo tumor growth and metastasis assay

The chick embryo tumor growth assay (INOVOTION, Grenoble, France) was performed as previously described[132], [133]. Briefly, Fertilized White Leghorn eggs, obtained from the Société Française de Production Agricole (SFPA, St. Brieuc, France), were incubated at 38°C with 60% relative humidity for 10 days. According to the French legislation, no ethical approval is needed for scientific experimentations using oviparous embryos (decree n° 2013–118, February 1, 2013; art. R-214–88). At stage E10, the chorioallantoic membrane (CAM) was dropped by drilling a small hole though the eggshell into the air sac and a 1 cm² window was cut in the eggshell above the CAM. Cultured MDA-MB-231-GFP were detached by trypsinization, washed with complete medium and suspended in serum free medium. A 50 µL inoculum of 1 x 10⁶ MDA-MB-231-GFP cells was added onto the CAM of each egg. Eggs were then randomized in 4 groups of 15 eggs (to get sufficient surviving embryos at the end of the experiments). One day later, tumors began to be detectable. They were then treated during 8 days, every two days (E11, E13, E15, E17), by dropping 100 µL of either carnosol (50 or 150 µ g/mL), colchicine (2 µ M) or vehicle (0.02% DMSO in PBS onto the tumor. At E19 the upper portion of the CAM was removed, transferred in PBS and the tumors were then carefully cut away
from normal CAM tissue and weighted. In parallel, a 1 cm² portion of the lower CAM was collected to evaluate the number of nodules, containing GFP-expressing cells. The fluorescent nodules were visualized in situ using whole mounts of tissue fixed in 4% formaldehyde in PBS and flattened between a hollow glass slide and a thick coverslip. In order to number the nodule, a thorough and complete visual scan of the piece of the lower CAM was done using Leica Macrofluo fluorescent microscope (Leica Microsystems GmbH) equipped with GFP filter. Chick embryos were sacrificed by decapitation.

2.10 Statistical analysis

All statistical analyses were performed using SPSS version 21. Data were reported as group mean ± SEM. The data were analyzed via one-way ANOVA. Significance for all statistical comparisons was set at p < 0.05.
Chapter 3: Results

3.1 Carnosol attenuates the migration ability of MDA-MB-231 human breast cancer cells

Cell migration is a crucial step in many physiological events. However, abnormal regulation of cell migration drives progression of various diseases, including cancer metastasis and invasion[134]–[136]. Understanding the fundamental mechanisms of breast cancer migration is essential for our understanding of both the pathology of disease and the effect of drug and its mechanism of action on breast cancer cells.

Therefore, we investigated the effect of carnosol on the migration ability of MDA-MB-231 cells through using wound-healing migration assay. For this purpose, we performed the test with two concentrations of carnosol (25 µM and 50 µM) and periods of treatment (0, 6 and 24 hrs) that were previously shown to be non-cytotoxic[128]. As shown in Figure 3A and 3B, carnosol treatment significantly inhibited cellular migration in a concentration- and time-dependent manner. Our data demonstrated that carnosol inhibits the migration potential of MDA-MB-231 cells.
Figure 3: Carnosol inhibits the migration of MDA-MB-231 human breast cancer cells. (A) Confluent culture of MDA-MB-231 cells were wounded by scratching with a pipette tip and the cells were incubated in DMEM medium supplemented with 10% fetal bovine serum without (control) and with indicated concentrations of carnosol. The wound was measured with an inverted microscope 40X magnification and photographed. (B) Wound healing assay showing that carnosol inhibited the migration of MDA-MB-231 breast cancer cells in time- and concentration-dependent manner. Values represent means ± SEM (n=3) distance (µm) that the cells have migrated in 6 and 24 hrs. Data are representative of three independent experiments.
3.2 Carnosol inhibits the invasion potential of MDA-MB-231 cells

Invasion of breast cancer cells into surrounding tissue is an initial step in tumor metastasis. For that reason, we examined the effect of carnosol on the invasive potential of MDA-MB-231 cells in Matrigel-coated Boyden chamber in the presence of vehicle (DMSO) or carnosol (25 µM). The number of carnosol-treated cells that has passed through the Matrigel coated membrane was markedly reduced in concentration-dependent manner by 40%, as shown in Figure 4A and 4B. This indicate that carnosol efficiently inhibits the invasiveness capability of MDA-MB-231 cells.
Figure 4: Carnosol inhibits the invasive activity of MDA-MB-231 cells. (A) MDA-MB-231 cells were incubated for 24 hrs with or without carnosol (25 µM) and LY294002 (20 µM). Cells that invaded into the matrigel were scored as described in Materials and Methods. DAPI-stained nuclei of invading cells were photographed at 100X magnification under an inverted microscope (Nikon Ti-U, Nikon). (B) Quantification of invaded MDA-MB-231 into the matrigel. Values represented in percent were calculated from three independent experiments and are represented as mean ±SEM. Statistical analysis was performed using one-way ANOVA (*p< 0.05, **p< 0.005, ***p< 0.001).
3.3 Carnosol suppresses the expression and the activity of MMP-2 and MMP-9 in MDA-MB-231 cells

Invasion of cancer cells into adjacent tissue requires the action of several hydrolytic enzymes or proteases (MMPs), which are released either by cells around the tumor or by tumor’s cells themselves[59]. MMP-9 and MMP-2, among other MMPs, are known to have a critical role in breast cancer cells invasion and metastasis[137]. Accordingly, we decided to examine by gelatin zymography assay, whether carnosol inhibits breast cancer cells invasion by affecting the activity of MMP-9 and MMP-2 enzymes. As it is shown in figure 5A, MMP-9 and MMP-2 activity was significantly reduced in concentration-dependent manner by carnosol.

Toward that, we examined the level of secreted MMP-9 in the conditioned medium of carnosol-treated MDA-MB-231 cells. The protein level of MMP-9 (Figure 5B) was found to be significantly reduced in response to carnosol treatment. Furthermore, RT-PCR analysis revealed that MMP-9 mRNA level was reduced in MDA-MB-231 cells upon treatment with carnosol (Figure 5C). This demonstrates that carnosol can inhibit the transcription of MMP-9 gene in these cells. Altogether, our results showed that carnosol significantly inhibits both, the expression and the activities of MMP-9 and MMP-2.
Figure 5: The effect of carnosol on MMP-2 and MMP-9 in MDA-MB-231 cells. (A) Activities of MMP-2 and MMP-9 in carnosol-treated MDA-MB-231 cells. Cells were treated with 25 and 50 µM carnosol for 24 hrs and then subjected to gelatin zymography, as described in Materials and Methods, to analyze the activities of MMP-2 and MMP-9. (B) Effects of carnosol on the secretion of MMP-9 in the collected conditioned medium of carnosol-treated MDA-MB-231 cells. The levels of secreted MMP-9 were determined using immunoassay kits as described in Materials and Methods. Experiments were repeated three times and the average of three means is represented ± SEM. Student’s t test was performed to determine the significance (*p< 0.05 and **p< 0.005). (C) Effects of carnosol on the expression of MMP-9 mRNA. MDA-MB-231 cells were treated with carnosol (25 and 50 µM) for 24 hrs and then subjected to RT-PCR to analyze the mRNA level of MMP-9. GAPDH was used as an internal control.
3.4 Carnosol down-regulates the expression of TNF-α protein in MDA-MB-231 Cells

Several studies reported that tumor necrosis factor-α (TNF-α) has particularly an important role in tumor migration, invasion and angiogenesis in various cancer including TNBC[138]. Hence, TNF-α should be considered as a target therapeutic approach for breast cancer treatment. Because we found that carnosol inhibited cell migration and invasion of MDA-MB-231, we sought to examine the effect of carnosol on the expression of TNF-α by measuring the protein level of this cytokine. Figure 6 shows that carnosol induced a marked reduction of TNF-α protein in MDA-MB-231 cells. Therefore, our data suggests that down-regulation of TNF-α could account, at least partly, for the inhibition of tumor growth and metastasis mediated by carnosol.
Figure 6: Effects of carnosol on the expression of TNF-α in MDA-MB-231 cells. Western blot quantification of TNF-α protein in carnosol-treated MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle (0.2% DMSO) or indicated concentrations of carnosol for 24 hrs, and then whole-cell extracts were subjected to Western blot analysis for TNF-α and β-actin proteins. Each lane was loaded with 25 µg of whole cell extracts. Loading was normalized to the levels of β-actin.
3.5 Carnosol attenuates STAT3 activation in MDA-MB-231 cells

Signal transducers and activators of transcription 3 (STAT3) gets activated by phosphorylation by various growth factor and is involved in mammary gland development[139]. STAT3 is classified as an onco-protein because its constitutive activation can mediate tumorigenesis in nude mice and oncogenic transformation in cultured cells[140]. Moreover, STAT3 activation can mediate cellular invasion due to the regulation of MMP-1 and MMP-2 which mediate cell invasion[141]. It also found to promote cellular proliferation, resistance to apoptosis, tumor angiogenesis, and migration of cancer cells[142].

Hence, STAT3 is widely recognized as a potential drug target for cancer therapy. To investigate whether carnosol affects the activation of STAT3 signaling in breast cancer cells, we analyzed the level of pSTAT3 in carnosol-treated MDA-MB-231 cells. We found that carnosol markedly reduced STAT3 phosphorylation, in concentration dependent-manner in MDA-MB-231 cells (Figure 7). This result suggests that the carnosol-mediated effect on cell proliferation, migration and invasion in breast cancer involves the inhibition of STAT3 signaling in breast cancer cells. Taken together, our data clearly indicate that carnosol exerts its effects on breast cancer cell proliferation, migration and invasion at least partly through an inhibition of one key signaling pathway, namely STAT3, known to regulate several processes such as tumor growth and metastasis in breast cancer.
Figure 7: Down-regulation of STAT3 in carnosol-treated MDA-MB-231 cells. Concentration-dependent decrease of phospho-STAT3 in carnosol-treated MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle (0.2% DMSO) or indicated concentrations of carnosol for 24 hrs, then whole-cell extracts were subjected to Western blot analysis for the phosphorylated form of STAT3 and for β-actin. Each lane was loaded with 25 µg of whole cell extracts. Loading was normalized to the levels of β-actin.
3.6 Carnosol inhibits tumor growth and metastasis in chick embryo tumor growth and metastasis assay

To further confirm the *in vitro* anti-breast cancer activities of carnosol, we decided to investigate its effect on tumor growth *in vivo* by using the chick embryo model. MDA-MB-231 cells were grafted on the chorioallantoic membrane (CAM) and formed tumors were treated every 48 h with vehicle, colchicine (2 µM) or increased concentrations of carnosol (50 and 100 µM). At E 19, tumors were recovered from the upper CAM and weighted. As it is shown in Figure 8A and 8B, carnosol significantly inhibited tumor growth compared with the vehicle treatment. In fact, concentrations of 50 and 100 µM carnosol led to reduced tumor growth by 43.6% and 33.8% respectively. Similar effect (43.6% inhibition) was obtained with 2 µM colchicine.

We next assessed for the ability of carnosol to inhibit metastasis by counting the number of nodules in the lower CAM in vehicle, colchicine and carnosol treated tumors. An average of 6.13 nodules were counted in the lower CAM of vehicle-treated chick embryo, while an average of 0.63 and 0.6 nodules only were counted in 50 and 100 µM carnosol-treated embryos (Figure 8C). All together, our data clearly demonstrates that carnosol could efficiently inhibit breast tumor growth and metastasis *in vivo*. 
Figure 8: Anti-tumor growth and anti-metastatic activity of carnosol on breast tumor in chick embryo chorioallantoic membrane model system. (A) MDA-MB-231 (1 x 10^6) cells were grafted on the CAM of 10 day (E10) chick embryo. Tumors were treated every 48 hrs with carnosol as described in Materials and Methods. At E19, tumors were collected and weighted. (B) Quantification of tumor weight in vehicle (DMSO), colchicine and indicated concentrations of carnosol-treated chick embryo. (C) Anti-metastatic effect of carnosol. Quantification of nodules observed in the lower CAM of chick embryo treated with vehicle (DMSO), colchicine or indicated concentrations of carnosol. Columns represents mean; bars represent SEM. Significantly different at ***p<0.0005.
Chapter 4: Discussion

TNBCs represent a challenge for patients and clinicians because of its poor prognosis and aggressive behavior, with lack of targeted therapies and high mortality in comparison to other subtypes of breast cancer. Therefore, identification of new effective therapeutic compounds to treat TNBCs remain an important clinical challenge.

Cancer progression toward metastasis involves multistep events in which malignant cells go into local invasion, intravasation, survival in the circulation, extravasation, colonization and angiogenesis[143]. Currently, most agents used for cancer treatment aim at blocking specific molecular mechanisms in tumor progression, such as, inducing cell death, blocking cell cycle progression or/and inhibiting tumor invasion and angiogenesis. Interestingly, numerous phytochemicals derived from edible plants have been reported to have anti-cancer activities[105]. However, little is known about the mechanism of action of most chemopreventive agents. In the present study we investigated the effect of carnosol, a naturally occurring compound, against TNBC specifically MDA-MB-231 cell line. Our findings demonstrate that carnosol, at non-cytotoxic concentrations, exerts a potent anti-metastatic and anti-tumor growth activities against the highly invasive MBA-MB-231 cell line. Indeed, we report that carnosol inhibited the migration and invasion of MDA-MB-231 cells. Moreover, carnosol suppressed the expression and activities of MMP-2 and MMP-9. Remarkably, our investigation revealed that TNF-α and STAT3 pathways were inhibited in MDA-MB-231 cells in response to carnosol, where both proteins are involved in invasion and metastasis. Furthermore, we
demonstrated that carnosol significantly inhibited tumor growth and metastasis \textit{in vivo} in chick tumor growth assay.

Metastases are formed following the completion of a complex succession of biological events and the earliest step is migration and invasion through the basement membrane and the extracellular matrix. Thus, to study this step of metastasis, we assessed the effect of carnosol on cell-ECM and cell-cell interactions and its effect on cells migration through \textit{in vitro} wound or scratch assay. Our results revealed that carnosol inhibited cell migration in the MDA-MB-231 cell line. This finding may account partially to the anti-metastatic potential of carnosol on TNBCs. For that reason, since carnosol exerts its anti-migratory effect on breast cancer cells, we speculated that it might be a valuable agent that can interfere with cancer cells adhesion to ECM.

Tumor invasion involves the entry of cancer cells that have resided within a well-confined primary tumor into nearby stroma and thereafter into adjacent normal tissue. Invasion is a crucial event in the process of tumor metastasis. It includes the secretion of substances to degrade the extracellular matrix and the basement membrane and also the regulation of protein expression involved in the control of cells motility and migration. MMPs are proteases that are capable of degrading a range of extracellular matrix proteins allowing cancer cells to migrate and invade\cite{144}, \cite{145}. Up to now, there are at least 28 identified MMPs, and 14 of them were found to be implicated with breast cancer development and progression\cite{146}. Indeed, the up-regulation of MMPs protein expression were found to be associated with breast cancer metastatic potential\cite{147}. Moreover, several studies of pathological mechanisms involved in tumor progression and metastasis showed
MMPs as prominent molecules involved in modeling tumor microenvironment and driving cancer progression and metastasis[148], [149]. Here, we clearly showed that carnosol exerts its anti-invasive effect by down-regulating MMP-2 and MM-9 expression and decreasing their activities. Therefore, through inhibiting these MMPs we might have impressive therapeutic effect against metastatic and invasive breast cancer.

Inflammation within breast tumor microenvironment is known to be associated with increased progression and invasiveness and poor prognosis[150], [151]. Many inflammatory cytokines are involved in the pathogenesis of breast cancer and they have gained increasing attention by researchers. TNF-α is considered as key inflammatory cytokine and plays a critical role in the pathogenesis of inflammatory responses, autoimmune and malignant diseases[152]. Furthermore, TNF-α stimulates the expression of adhesion molecules included in the invasion of metastatic tumor cells[153], [154]. Additionally, TNF-α is also a vital molecule in stimulation of angiogenesis through promotion of endothelial cell proliferation and improvement of expression of other pro-angiogenic factors[155]. Further, in vitro activation of TNF-α induces malignant behavior and invasion in breast cancer cells[156]. Hui-Hui Li et al. suggested that up-regulation of TNF-α expression in TNBC’s patients is associated with distant tumor metastasis more than other breast cancer subtypes[157]. In contrast, the knockdown of TNF-α gene expression was found to be associated with the inhibition of cell proliferation and induced apoptosis in TNBC[158]. Likewise, inhibition of tumor xenografts in MDA-MB-231 cells by docetaxel and ulinastatin was correlated with down-regulation of TNF-α expression and other cytokines expression[159]. Moreover, El Hasasna et al. found that Rhus
coriaria suppressed TNF-α production in MDA-MB-231 breast cancer cells[160]. Interestingly, here we showed that carnosol inhibited TNF-α expression in MDA-MB-231 cells. Taken together, our finding strongly suggests that one possible mechanism through which carnosol inhibits invasion and tumor growth involves inhibition of TNF-α pathway.

STAT3 is a cytoplasmic transcription factor that is responsible for signal transduction from cell surface receptors to the nucleus. STAT3 activation is tightly controlled under normal physiological conditions[142], [161]. However, compelling evidence suggest that STAT3 is a master regulator of several key hallmarks of cancer cells and is found to be constitutively activated in many cancers including breast cancer[7], [9], [162], [163]. This transcription factor plays a pivotal role in tumor growth, cellular proliferation[164], resistance to apoptosis regulators[165], invasion, migration and angiogenesis[166], [167]. For example, in transgenic mice, mutant allele of constitutively active STAT3 developed significantly more metastatic and aggressive tumors than those without[168]. Conversely, Kotha et al. reported that resveratrol, a naturally occurring polyphenolic compound, repressed tumor growth and induced apoptosis by inhibiting STAT3 signaling in malignant cells like human breast cancer (MDA-MB-468 and MDA-MB-231), prostate cancer, human pancreatic cancer containing activated STAT3 protein[169]. Moreover, Methylsulfonylmethane (MSM), an organic sulfur-containing natural compound, has been shown to suppress breast cancer growth by downregulating STAT3 and STAT5b pathways[170]. Remarkably, in MDA-MB-231 cells, we showed that carnosol inhibited STAT3 production in dose-dependent manner. Thus, STAT3 is an attractive target for anti-breast cancer drug development.
Metastasis is the most lethal aspect of cancer where it involves complex events that are linked both temporally and spatially. Since therapeutic failures are usually due to an inability to stop the invasion and spread of cancer rather than to failure to treat the primary tumor. Experimental methods for studying the metastatic properties of tumor cells are essential to design the most favorable therapy and for a basic understanding of the mechanism(s) of tumor progression. The chick embryo affords a powerful model to assess tumor metastasis in vivo, in which it provides accessibility to the chorioallantoic membrane (CAM), a well-vascularized tissue that surrounds the chick embryo. This model is inexpensive and convenient and it has other advantages: (1) it is immunodeficient, thus tumors will be able to form after transplantation onto CAM, (2) invasion and attraction of blood vessels are crucial for tumor growth and this can be easily observed in CAM as it surrounds the embryo[171], [172]. Therefore, to extend our previous findings we utilized the chick embryo CAM metastasis assay to model the metastatic process and the affect of carnosol in vivo. Human breast cancer cells (MDA-MB-231) were transplanted into CAM’s chick embryo and treated with carnosol. After 19 days, tumors were recovered from the upper CAM and weighted and nodules in the lower CAM were counted. Interestingly, we found that carnosol inhibits tumor growth and metastasis in vivo. All together, our data demonstrate that carnosol could efficiently inhibit breast tumor growth and metastasis in vivo.

In summary, our data are consistent with a model shown in Figure 9. We demonstrated, for the first time, that carnosol exerts a potent anti-invasive, anti-metastatic and anti-growth effects against the highly proliferative and invasive human breast cancer cell line (MDA-MB-231). Carnosol modulate the expression
and/or the activity of proteins that regulate cellular migration, invasion and angiogenesis such as MMP-9, MMP-2, TNF-α and STAT3. Moreover, this study demonstrated that carnosol inhibited tumor growth and metastasis in an in vivo tumor growth and metastasis assay. Therefore, our current findings provide the first instance of a potential role of carnosol as promising chemopreventive and therapeutic agent that inhibits breast cancer growth and metastasis by modulating the expression and activities of several target proteins and/or pathways. Currently, there is a growing interest in identifying natural derived compounds that affect several targets or pathways in breast cancer. Thus carnosol certainly merits a lot of attention and further study to identify its downstream mechanisms of action.

Figure 9: Hypothetic model demonstrating the differential effect of carnosol in MDA-MB-231 triple.
Chapter 5: Conclusion

Chemoprevention by edible phytochemicals is now believed to be an inexpensive, acceptable, readily applicable and accessible approach for cancer control. Various phytochemicals derived from edible plants have been shown to interfere with different stages of tumorigenesis. Many cellular processes and mechanisms have been shown to account for the anti-carcinogenic actions of dietary components, but attention has recently been focused on intracellular signaling cascades as common molecular targets for various chemopreventive phytochemicals.

Here, our study has focused on carnosol, a natural compound found in our diets, as a potential anti-breast cancer agent. We demonstrated that carnosol attenuated breast cancer cell migration, invasion and tumor growth in vivo, at least partially, through down-regulating the expression of TNF-α and STAT3 which ultimately led to the inhibition of MMP-2 and MMP-9 expression and activity.

Nowadays, increasing number of natural products are being evaluated in intervention trials for their potential as anti-cancer agents. Even with the major advances in our understanding of multi-step tumorigenesis, little is known regarding the mechanism of action of most chemopreventive candidates. The anti-cancer effect of most dietary phytochemicals are likely to be the sum of several different mechanisms. Deregulation or disruption of intracellular signaling pathways frequently lead to malignant transformation of cells. Therefore, it is important to identify molecules in the signaling cascade that can be affected by phytochemicals to permit better assessment of their underlying mechanisms. Therefore, naturally occurring phytochemicals are indeed worthy of further assessment and development as anti-metastatic and anti-cancer agents for clinical use.
Bibliography


List of Publications
