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# MOLECULAR MECHANISM OF ACTION OF THE NATURAL POLYPHENOLIC COMPOUND AND THE P300 INHIBITOR "CARNOSOL" AGAINST THE TRIPLE NEGATIVE BREAST CANCE

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This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Professor Rabah Iratni

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## Declaration of Original Work

I, Halima Ali Mohammed Salem Alsamri, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Molecular Mechanism of Action of the Natural Polyphenolic Compound and the P300 Inhibitor "Carnosol" Against the Triple Negative Breast Cancer*", hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Professor Rabah Iratni, in the College of Science at UAEU. This work has not previously 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions.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 dissertation.

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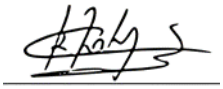
   Signature  Date 15.11.2021

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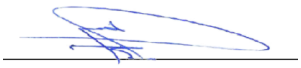
   Signature  Date 20.11.2021

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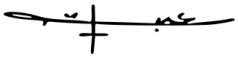
   Signature  Date 15.11.2021

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## Abstract

Carnosol, a naturally occurring phytopolyphenol found in sage, oregano and rosemary, has been extensively studied by our laboratory for its anticancer effects in various types of cancer. In human Triple Negative Breast Cancer (TNBC), carnosol was shown to inhibit cellular viability, colony growth, induced cell cycle arrest, autophagy, and apoptosis. Nonetheless, very little is known about molecular mechanism of action. In the current study, the ability of carnosol to inhibit metastasis and tumor growth was examined. Wound healing and invasion assays revealed that carnosol inhibited migration and invasion at non-cytotoxic concentrations of MDA-MB-231 cells. Also, carnosol found to inhibit the activity and downregulated the expression of MMP-9. Activation of STAT3, a transcription factor that regulates MMP-9 expression, was also inhibited via carnosol-mediated ROS-dependent proteasome degradation. *In vivo* study using chick embryo tumor growth assay has shown that carnosol significantly and markedly suppressed tumor growth and metastasis of breast cancer xenografts. Additionally, we found that carnosol induce ROS-dependent, p38-dependent ER stress and activates UPR via upregulating the ER stress sensors (ATF4/CHOP, ATF6α, and IRE1α/XBP1). Also, upstream triggers of Unfolded Protein Response (UPR) pathway, β-catenin and ER stress chaperones, were upregulated. On the other hand, cell survival Akt/mTOR signaling pathway was downregulated in a ROS-dependent manner. We also found that carnosol targeted p300 and PCAF Histone Acetyltransferases (HATs) to proteasome degradation through a ROS-dependent mechanism. Interestingly, using a cell-free system, we show for the first time that carnosol efficiently and selectively inhibited histone acetyltransferase activity of p300 while having no effect on the other HATs such as PCAF and GCN5. This work provides further confirmation that carnosol represents a promising anti-breast cancer therapeutic compound and identifies it as a novel natural p300 inhibitor that could be added to the existing panel.

**Keywords**: TNBC, metastasis, tumor growth, STAT3, ROS, UPR, p300, acetyltransferase activity.

## Title and Abstract (in Arabic)

# آلية العمل الجزيئية للمركب الطبيعي المتعدد الفينول ومانع P300 "كارنوسول" ضد سرطان الثدي السلبي الثلاثي


***الملخص***

الكارنوسول هو مركب فيتوبوليفينول موجود طبيعياً في بعض النباتات كالمرمية والزعتر وإكليل الجبل، تمت دراسة تأثيره على أنواع مختلفة من السرطانات. في سرطان الثدي الثلاثي السلبية (TNBC)، ثبت أن الكارنوسول يثبط تكاثر الخلايا السرطانية ونمو المستعمراتها، كما أنه يوقف دورة الخلية ويؤدي إلى موتها إما بالالتهام الذاتي او الموت المبرمج. ومع ذلك، لا يُعرف سوى القليل جدًا عن آلية عمل الكارنوسول الخلوية والجزيئية. في هذه الدراسة الحالية، تم تقييم قدرة الكارنوسول على تثبيط انتقال الخلايا السرطانية من عضو إلى أخر(metastasis) وأيضاً نمو الورم الخبيث، كما أنه تمت دراسة تأثير الكارنوسول ونقص الحمض الميني الأستيل على بروتينات الهيستون في المختبر والجسم الحي. كشفت التجارب المختبرية في الدراسة الحالية الكارنوسول قادر على تثبيط انتقال وغزو الخلايا السرطانية وأيضاً نمو الورم الخبيث عن طريق تجربة ألتام الجرح وغزو الجيلاتين. إضافة إلى ذلك، كشفت تجارب الجيلاتين زيموغرافي (gelatin zymography) والانزيم المرتبط بالمناعة (ELISA) والنسخ العكسي لمتفاعل المتسلسل البوليميرازي (RT-PCR) أن الكارنوسول قادر على تثبيط تعبير ونشاط MMP-9. بروتين معامل الاستنساخ STAT3 له دور كبير في التحكم في التعبير البروتيني ل MMP-9، لذلك تم اختبار تأثير الكارنوسول عليه وأظهرت النتائج انه تعبير ال STAT3 تم تثبيطه بواسطة تواجد الاكسجينات التفاعلية ROS المحفزة بواسطة الكارنوسول. إضاقة إلى ذلك، تم اختبار الكارنوسول على نمو الورم الخبيث في أجنة الفراخ، وأظهرت النتائج قدرة الكارنوسول على إحباط نمو الورم بشكل ملحوظ. وفي الجزء الآخر من البحث، أثبت الكارنوسول قدرته على إحداث إجهاد الشبكة الاندوبلازمية (ER-stress) الذي يؤدي إلى تفعيل مسار الاستجابة للبروتينات المتدمرة UPR عن طريقة ازدياد التعبير البروتيني ل ,ATF4/CHOP ATF6αو IRE1α /XBP1. علاوة على ذلك، β-catenin والبروتينات المرافقة لإجهاد الشبكة الاندوبلازمية chaperones، تم ثبيط تعابيرهم البروتينه بواسطة الكارنوسول. وفي الجهة المقابلة قام الكارنوسول بتخفيض الإشارات الحيوية عن طريق ثبيط بروتينين Akt / mTOR. كما اثبتت التجارب أن الكارنوسول قادر على تنظيم موت الخلايا السرطانية عن طريقة تحفيز التعبير البروتيني لP38 . وفي الجزء الأخير من الدراسة أظهر الكارنوسول قدرته على تحفيز

نقص الحمض الميني الأستيل على بروتينات الهيستون عن طريق إحباط البروتينات المسؤولة كال P300 وPCAFخصيصاً وذلك اعتماداً على تواجد الاكسجينات التفاعلية ROS. وفي الختام تقدم هذه الدراسة دليلاً على أن الكارنوسول قد يمثل مرشحًا علاجيًا واعدًا يعالج انتشار ونمو ورم سرطان الثدي وكمثبط طبيعي جديد كال P300.

**مفاهيم البحث الرئيسية**: سرطان الثدي الثلاثي السلبية، نمو الورم الخبيث، STAT3، الاكسجينات التفاعلية، p300، إجهاد الشبكة الاندوبلازمية، الحمض الميني الأستيل.

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# Dedication

*To my father's soul and beloved mother*

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# List of Abbreviations

| | |
|---|---|
| ATF4 | Activator of Transcription Factor 4 |
| ATF6 | Activator of Transcription Factor 6 |
| CHOP | CCAAT/Enhancer-Binding Protein Homologues Protein |
| ER | Endoplasmic Reticulum |
| HAT | Histone Acetyltransferase |
| IRE1α | Inositol Requiring Enzyme 1α |
| MMP | Matrix Metalloproteinases |
| mTOR | Mammalian Target of Rapamycin |
| PERK | Protein Kinase R-like Kinase |
| ROS | Reactive Oxygen Species |
| STAT3 | Signal Transducer and Activator of Transcription 3 |
| TNBC | Triple Negative Breast Cancer |
| UPR | Unfolded Protein Response |
| XBP1 | X-box Binding Protein 1 |

# Chapter 1: Introduction

## 1.1 Breast Cancer

Over the past five decades, deaths from heart disease, stroke, and pneumonia have dropped because of the newly developed treatments and preventive approaches based on a profound understanding of these diseases' risk factors and pathogenesis. At the same period, cancer mortality relatively has not changed [1]. We are currently at a turning point in history in which cancer deaths exceed those from cardiovascular diseases [2,3].

Cancer is a multifactorial disease that begins when cells divide uncontrollably and spread into nearby tissues [5]. In 2020, breast cancer was the leading cause of death among women globally. Breast cancer remains the fifth most common cancer in terms of incidence and mortality, presenting 11.7% of total incidence and the 6.9% of total deaths [6,7]. In the United Arab Emirates, breast cancer presents 21.4% (1030 out of 4807) of all cancer types and 11.8% (222 out of 1896) of cancer deaths [8]. The recorded number of cases in 2020 was 1030 and is expected to increase to 2310 by 2040. The burden of breast cancer incidence and mortality is obviously increasing, exceeding all other cancers worldwide [9]. Therefore, it is urgently needed to discover efficient treatments for breast cancer.

Breast cancer is a heterogeneous disease, which makes it challenging to diagnose and treat [9]. Currently, breast cancer is mostly classify based on the proliferation index (Ki67) and expression of hormone receptors, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Accordingly, there are four molecular subtypes of breast cancer which are luminal A

($ER^+$ /$PR^+$ /Ki67 low <14% or Ki67 intermediate 14–19%), luminal B ($ER^+$ /$PR^+$ /$HER2^{-/+}$ /Ki67 intermediate 14–19% or Ki67 high > 20%), basal-like or triple-negative ($ER^-$/$PR^-$/$HER2^-$/Ki67) and HER2 overexpressing ($ER^-$/$PR^-$/$HER2^+$/Ki67) [10–13].

### 1.1.1 Triple-Negative Breast Cancer

Among these, TNBC that lack the expression of the three main hormonal receptors, accounts for approximately 15% to 20% of all breast cancer types [14], frequently occur in younger (<50 years) women and are more prevalent in Hispanic and African American women [15]. TNBC exhibits increased proliferation markers, mitotic activity, high-grade nuclear atypia, scant stromal content, high nuclear cytoplasmic ratio, central necrosis, multiple apoptotic cells, invasive, and stromal lymphocytic infiltration [16,17]. Moreover, TNBC is characterized by a rapid growth rate, higher grade compared to other breast cancers, lymph node progression, and can metastasize to other organs, mostly, lungs and brain [18]. TNBC was also found to have a poor prognosis, very aggressive, high reoccurrence rate, and shorter survival period [9,10,19,20]. This is because it lacks the hormonal receptors' expression, thus cannot be efficiently targeted by endocrine therapy (e.g., tamoxifen, aromatase inhibitors) and HER2-directed therapies (e.g., trastuzumab) [21]. TNBC is a heterogeneous disease by itself, constitutes different molecular subtypes that can vary in response to therapy. The current treatment of TNBCs mainly relies on chemotherapy; however, this treatment face resistances and metastasis occurs [22]. Consequently, this makes it challenging for the clinical management and current treatments. Hence, new targeted therapeutic approaches for TNBC are an unmet need.

### 1.2 Phytochemicals with Therapeutic Potential Against TNBC

TNBC is the most aggressive form of breast cancer, and the incidence of new cases and mortality is rising worldwide. Due to the lack of specifically known targets and targeted treatments, TNBC develops a high tendency to drug resistance and metastasize to lung, bone, and brain, making it very challenging to treat [23,24]. Currently, TNBCs are treated with chemotherapy agents alone or in combination with radiotherapy or surgery [25]. The current use and approved chemotherapeutic agents for TNBC include cisplatin, anthracycline, taxanes, paclitaxel, tamoxifen, and platinum compounds [22,26,27]. However, besides the other conventional therapies, these chemotherapeutics approaches made the patients suffer from severe side effects, and develop resistance to them with time [28]. Identifying a new novel anticancer drug to treat TNBCs is relatively considered an expensive approach. Henceforward, we observed that the current focus is leaning towards using plant-derived compounds (also called phytochemicals) treatment options. Because they are more convenient to use, have lower cost, exhibit less toxicity, more effective, and with reduced side effects, thus, the use of phytochemicals for treating TNBC patients is a promising option.

Phytochemicals have been used for medicinal purposes since the origin of humanity. Due to the convenient and beneficial properties of phytochemicals (like effective, cheaper, and less toxic), employing them for TNBC therapy can be a vital strategy and improve the life quality of TNBC's patients [29]. Several phytochemicals displayed anticancer activity against TNBC and other cancers, such as resveratrol, indole-3-carbino, fisetin, 6-gingerol, curcumin, capsaicin, and quercetin. This is because they target several molecular pathways in carcinogenesis, including apoptosis, proliferation, cancer stem cells, inflammation, invasion, metastasis, angiogenesis, and

the likes [30–32]. Specifically, through the alteration of multiple cellular pathways like TNF-α, Notch, Wnt-ß, TGF-ß, Ras, EGFR, INF-γ, and hedgehog which are implicated in tumor development [33]. Several researchers reported promising anticancer activity of phytochemical either alone or in combination with a chemotherapeutic drug.

The increased virulence of triple-negative tumors might be attributed to the increases in the expression and activity of vascular endothelial growth factor (VEGF). VEGF is a major angiogenic factor known to promotes tumor growth and metastasis, leading to higher morbidity [34,35]. Hence, VEGF-stimulated proliferation and migratory pathways make it a good target of adjunctive chemotherapy. Luteolin is a phytochemical (a flavonoid commonly found in many vegetables, fruits, and medicinal herbs) that inhibits VEGF expression and suppresses the proliferation of several cancer cells. It can also restrain invasion, metastasis, angiogenesis, reverse epithelial-mesenchymal transition (EMT) and induce apoptosis [36–38]. For that reason, Cook *et al.* evaluated the effect of luteolin against TNBC and studied its potential to reduce metastasis and tumor growth. *In vivo* studies showed that luteolin suppresses lung metastasis of triple-negative cell lines (MDA-MB-435 and MDA-MB-231) to the lung, in which metastasized TNBC colonies were reduced from ~14% to ~5%. Further, the *in vitro* experiment revealed that luteolin significantly reduced cell viability at a concentration > 10 $\mu M$, induced apoptosis, and VEGF secretion was significantly inhibited [39].

Morover, several studies have reported promising synergistic effects of combining phytochemical with the chemotherapeutic agent to treat cancer. In that case, piperine is an alkaloid phytochemical, which shows anticancer activity through suppressed TNF-α mediated cellular processes, activate NF- Kb and expressed cell adhesion molecules, and blocked VEGF mediated tumor angiogenesis [40,41]. Henceforth, due

to piperine's broad spectrum of anticancer activity, this makes it a potent adjuvant to be used in combination with other anticancer chemotherapeutic agents [42]. This is confirmed when Abdelhamed *et al*. screened many phytochemicals along with TRAIL therapeutics, studying their synergistic activity against TNBC cell lines (MDA-MB-468, MDA-MB-231, and 4 T1 [murine]) [42]. Piperine was found to be the best adjuvant candidate in combination therapy, in which it improves the efficacy of TRAIL therapeutics against TNBC. The combination of piperine and TRAIL showed more efficient induction of cytotoxicity, apoptosis, and cell cycle arrest than using piperine or TRAIL alone to treat TNBC [42]. Another study by Şakalar *et al*. investigated the anticancer effect of combining the phytochemical thymoquinone (the active ingredient in *Nigella sativa*) with the chemotherapeutic drug paclitaxel against the mammary triple negative cells 4T1. Their data suggest that combination therapy of thymoquinone + paclitaxel showed significant synergistic cooperation, in which thymoquinone sensitizes 4T1 cells to paclitaxel through several signaling involving p53, apoptosis, and tumor suppressor genes [43].

Therefore, considering discussed studies above, phytochemicals alone or in combination with other chemotherapeutic drugs have substantial potential in targeting numerous cascades in TNBC tumors. Exploring and identifying phytochemicals besides the development of other therapeutics advances can serve as potential tools in the future to decimate TNBC. Next, a promising phytochemical, carnosol, and its potential to treat TNBC will be discussed.

### 1.3 Carnosol

Dietary phytochemicals have been appreciated to prevent and cure several lethal diseases, including cancer [43]. Plant bioactive moieties include xanthonoids,

flavonoids, and terpenoids, in which terpenoids are considered one of the most significant classes [44]. Carnosol is a polyphenolic phytochemical that belong to the terpenoids class and is known to possess a range of therapeutic effects such as anti-inflammatory [45], antioxidant [46], neuroprotective [47], antimicrobial [48], and anticancer properties [49,50]. Talking about cancer, several studies have revealed the anticancer effects of carnosol are through the inhibition of proliferation and survival, restrain migration and invasion, and enhance apoptosis [50,51(p. 2),52]. All these anticancer effects are mediated via modulating various signaling pathways, including apoptosis (Bax/Bcl2/caspases), survival and proliferation (Akt/mTOR/MAPK), transcription factors like NF-κB, STAT3-6, and other cellular pathways. The triple-negative tumor is one of the most aggressive and hard to treat, and lately, few studies have reported the effect of carnosol against TNBC. Here, the current section (1.3) will highlight the source and chemical characterization (1.3.1), safety and toxicity (1.3.2), and existing evidence examining the anticancer effects of carnosol against TNBC (1.3.3).

### 1.3.1 Carnosol's Sources, Chemistry and Structural Characterization

Rosemary and sage are Mediterranean herbs used for culinary purposes and have been known to contain various bioactive compounds, including polyphenols such as carnosol, carnosic acid rosmanol, rosmarinic acid as well as others. Carnosol was firstly isolated from sage (*Salvia carnosa*) in 1942, and its chemical structure ($C_{20}H_{28}O_4$) was first identified by Brieskorn *et al.* in 1964 [53]; Figure 1 illustrates the carnosol structure and one example of plant source, sage. Carnosol, an ortho-diphenolic diterpene with an abietane carbon skeleton with hydroxyl groups at positions C-11 and C-12 and a lactone moiety across the B ring and considered as result product from the oxidative degradation reaction of carnosic acid [54,55]. Its

absorbance corresponds to $\lambda_{max}$ = 212**,** 285 nm. Carnosol is soluble in organic solvents such as dimethyl sulfoxide (DMSO), ethanol, and dimethyl formamide, in which solubility in these solvents is 250, 8, and 35 mg/ml, respectively [54]. Nowadays, carnosol is receiving increasing attention for its various health-promoting properties.

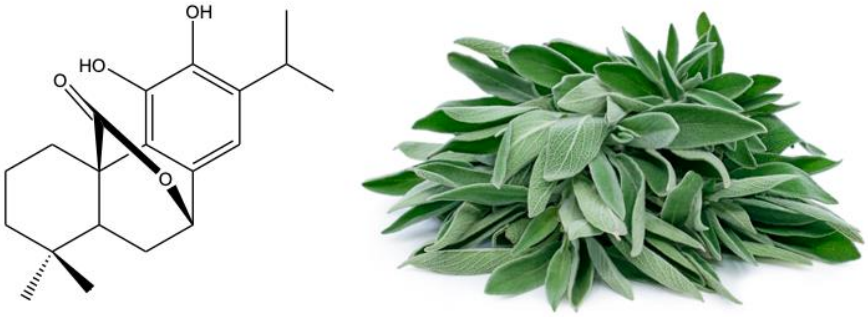


Figure 1: Chemical structure of carnosol (left) found in sage (right).

### 1.3.2 Safety and Toxicological Studies on Carnosol

Toxicological studies are essential to develop therapeutic drugs for clinical use. Several animal studies have suggested that the daily oral intake of carnosol is safe. Johnson *et al*. revealed that carnosol at a dose of 30 mg/kg was well tolerated in mice [49]. Similarly, Sprague–Dawley rats were fed with an AIN-76A diet supplemented with 1% carnosol for two weeks, and it showed no side effects on body weight [56]. Agreeing with previous studies, Phipps *et al*. recently investigated the toxicity of carnosol and carsonic acid using rosemary extract for 90 days in males and females mouse models, and they found no adverse side effect for a high dose reaching 195 mg/kg body weight/day which is equivalent to 64 mg/kg body weight/day carnosol and carnosic acid [57].

Traditional herbal medicine has good therapeutic values; FDA has recognized carnosol-enriched rosemary extracts as “Generally Recognized as Safe” (GRAS). This study tested the toxicity of several herbal extracts, including rosemary, the dose range was between 80 to 400 mg/kg/bw/day, which was equivalent to 20–60 mg/kg/day of carnosol and carnosic acid per day, and there were no observable adverse effects on the used animal models [58].

### 1.3.3 Effects of Carnosol on TNBC

Triple-negative is the most complicated to treat and the most fatal breast cancer type, and the median of survival for metastatic patients is around one year [59]. The phytochemical, carnosol, showed promising health-promoting activities [60] and there are few studies in the literature review that have been demonstrated the anticancer effects of carnosol on TNBC. Generally, carnosol has been reported to affect TNBCs through reducing cellular viability, inducing apoptosis and autophagy, block the cell cycle, inhibiting migration and metastasis, preventing tumor growth, and affecting post-translational modification via inhibiting p300 acetyltransferase. Table 1 summarize studies that reported the effects of carnosol on TNBC.

Table 1: Summary of studies that reported the effects of carnosol on TNBC.

| Cell line | Carnosol alone or in combination (dose & duration) | | Experimental model | | | Mechanism of action | Targeted proteins | Ref. |
|---|---|---|---|---|---|---|---|---|
| | Alone | In combination | *In vitro* | *In vivo* | *In silico* | | | |
| MDA-MB-231 | 10–100 μM; 2–12 h | - | ✓ | - | - | Carnosol impaired the proliferation through CYP1A1 reduction and AMPK activation. | ↓ CYP1A1<br>↑ pAMPK<br>↓ AhR | [61] |
| HBL-100<br>MDA-231<br>MDA-435 | 12.5–200 μM; 4 h, 24 h, 48 h & 72 h | Carnosol 50 μM + Curcumin 70 μM; 4 h | ✓ | - | - | Carnosol alone inhibited the viability of all TNBC cell lines. The combination with curcumin was tested using MDA-231, which resulted in a synergistic reduction in vitality, apoptosis induction, and cell cycle blockade. | ↓ Cyclin D1<br>↓ Bcl2<br>↓ Survivin<br>↑ p27 | [62] |
| MDA-MB-231 | 25–100 μM; 24 h & 48 h | - | ✓ | - | - | Carnosol reduced viability, block cell cycle at G2 phase, induce DNA damage, reduce the mitochondrial potential, cause ROS-dependent apoptosis intrinsically and extrinsically, induce beciln-1-independent autophagy, and constrain colony growth. | ↑ p21/WAF1<br>↓ p27<br>↑ PARP<br>↑ Caspases 3,8,9<br>↓ Bcl2/ ↑ Bax<br>↑ LC3II<br>↓ p62 (SQSTM1)<br>↑ pERK1/2<br>↑ γH2AX | [50] |

Table 1: Summary of studies that reported the effects of carnosol on TNBC (continued).

| Cell line | Carnosol alone or in combination (dose & duration) | | Experimental model | | | Mechanism of action | Targeted proteins | Ref. |
|---|---|---|---|---|---|---|---|---|
| | Alone | In combination | *In vitro* | *In vivo* | *In silico* | | | |
| MDA-MB-231 | EC50 < 9 μM | | ✓ | | | Carnosol showed an antiproliferative effect | - | [63] |

### 1.3.3.1 Carnosol Induces Cell Cycle Blockage

Cell cycle is a highly regulated and organized process that responsible for cellular proliferation and growth via ensuring the duplication of genetic material and cell division. This process comprises growth-regulatory signals as well as checkpoints proteins that monitor the genetic integrity to ensure the absence of any genetic damage. Cells reproduce through the progression into four distinct phases of the cell cycle; G0/G1, S, G2 and M. Theses phases are controlled by a number of CDKs, that act in complex with their cyclin partners (cyclin D, E and B), drive the cell forward through the cell cycle. However, whenever an error exists in any of the cell cycle phases, CDK inhibitors interfere to stop the cycle's progression, either to fix it or to induce cell death. The INK4 is class of CDK inhibitors, includes p16, p15, p18, and p19, which bind and inhibit cyclin D–associated kinases such as CDK4, and 6. Another class of CDK inhibitors is the kinase inhibitor proteins (KIP), involves p21, p27, and p57, which negatively regulate cyclin E and A to bind to CDK1 CDK2 complexes [64,65].

Aberrant cell cycle activity and uncontrolled cellular proliferation are characteristics of cancer cells. These results from mutations in upstream signaling pathways or genetic lesions occur in genes encoding cell cycle proteins. Also, human cancers are

seen with aberrant activation of CDKs [66], therefore, finding inhibitors of CDKs and cell cycle regulators are considered attractive targets in cancer therapy.

Most of the currently used anticancer drugs (like; tamoxifen and methotrexate [67]) limit tumor progression by hindering one or more cell cycle phases and their transition points, and consequently attenuate the cancer cell proliferation. Arresting of the cell cycle by these drugs may also be associated with the induction with apoptosis [68]. Importantly, several studies claimed that the anticancer effects of carnosol is via the inhibition of cancer cell proliferation either via cell cycle arrest or induction of apoptosis [69,70]. In a study utilizing TNBC cell line, MDA-MB-231, Al Dhaheri *et al*. checked whether carnosol cytotoxic mechanism of action affect the cell cycle distribution, the analysis showed that carnosol (50 and 100 µM) induced G2 cell cycle blockade, this was confirmed with an overexpression of p21 (a CDK inhibitor) [50]. Carnosol showed promising anticarcinogenic effects in triple-negative and other cancers, yet further investigations are needed through utilizing *in vivo* animal models in addition to the clinical studies to validate the usefulness of carnosol against cancer.

#### 1.3.3.2 The Role of Carnosol in Inducing Cell Death Mechanisms

##### 1.3.3.2.1 Carnosol Induces Apoptosis

Apoptosis is a type of programmed cell death that plays a vital role in maintaining tissue homeostasis by eliminating damaged and unwanted cells. Apoptosis is an evolutionarily conserved and highly regulated energy-dependent process that happens discretely in individual cells of our body [71]. However, deregulation of apoptosis is implicated in several pathological conditions, including cancer. Usually, cancer cells resist and escape apoptosis by over-expressing the antiapoptotic proteins and

repressing tumor suppressor genes. Consequently, this led to cancer development, progression, tumor growth, and develop treatment resistance [72,73]. TNBC is one of the most aggressive cancer types known to evade apoptosis and develop resistance to currently used therapeutic approaches (like; chemotherapy and radiotherapy). Even though current treatments are acting via activating cell death pathways like apoptosis, TNBC finds a way to relapse and develop resistance toward the treatment and apoptosis, making it clinically challenging to treat and find targeted treatment for triple-negative tumors [74]. Henceforth, a better understanding of the molecular mechanisms underlying TNBC resistance to apoptosis will probably provide the basis for a rational method to develop targeted molecular therapies.

Carnosol is one among the polyphenols reported to have anticancer effects against several kinds of human cancer cell lines and animal models [75]. Due to the high challenges facing treatment for triple-negative, this attracted the interest of Al Dhaheri's team to investigate the anticancer properties of carnosol on the TNBC cell line, MDA-MB-231 [50]. They have started their investigation by examining the viability of MDA-MB-231 cells upon the treatment with different concentrations (25, 50, and 100 μM) of carnosol for 24 h and 48 h, and results showed that carnosol exhibited antiproliferative activity in concentration- and time-dependent manner. Next, they inspected whether carnosol reduced viability via the induction of the apoptosis pathway. Annexin V staining (detect apoptotic cells by binding to phosphatidylserine), activation of intrinsic apoptosis (cleaved PARP and caspase 3), and extrinsic apoptosis (caspase 8 and 9) pathways were detected in the high concentration conditions only (50 and 100 μM) at 24 h. Moreover, carnosol had modulated the expression of Bcl-2/Bax and induced mitochondrial membrane

potential depolarization, which is associated with the activation of apoptosis observed in the MDA-MB-231 cells [50].

#### 1.3.3.2.2 Carnosol Induces Autophagy

Autophagy is another mechanism that serves to maintain cellular homeostasis via dragging damaged or unwanted cellular components to the lysosome, either to get rid of it or recycle it and results in promoting cellular survival. In this way, cancer cells can use autophagy to prevent the accumulation of cellular damages caused by chemotherapeutics, promoting temporal survival and developing chemoresistance [76]. Several pieces of evidence have indicated that autophagy is involved in cancer cells' adaptation to chemotherapy [77,78]. This suggests that when the tumor is undergoing chemotherapy, most often activate autophagy as a cytoprotective mechanism to survive the effects of anticancer drugs, which, sequentially, may drive chemoresistance. Contrary to its cytoprotective role, autophagy is also known to cause cell death stimulated by excessive cellular stress, for that it is called; type II programmed cell death [79]. More importantly, cell death mediated by autophagy can involve the stimulation of apoptosis [80,8]. In contrast, inhibiting autophagy has been detected to reduce apoptosis in some tumors [82]. In this regard, both cell death mechanisms apoptosis and autophagy may crisscross with each other and share common actions to derive cellular death response.

This controversial role of autophagy derives Al Dhaheri and coworkers to investigate the existence of autophagy in TNBC upon the treatment with carnosol. In the begining, they observed under light and electron microscopy the presence of cytoplasmic vacuolation is present, which might be associated with autophagy induction. To further

confirm the presence of autophagy, they assessed first the conversion of LC3I (cytosolic form) into a lipidized LC3II (autophagosome membrane-bound form) which is one of the characteristics of autophagy initiation has. Carnosol was found to induce the accumulation of LC3II in a concentration-dependent manner. Another widely known autophagy marker was evaluated is p62 (SQSTM1), a ubiquitin-binding protein whose level decreases when autophagy flux increases. At a lower concentration of carnosol (25 μM), there was a slight increase in p62 (SQSTM1) expression; nonetheless, at higher concentrations (50 and 100 μM), where massive autophagy exists, p62 (SQSTM1) decreases. Next, they assessed the expression of Beclin-1 (responsible for autophagosome formation, a step in forming autophagic vacuoles). Beclin-1 remained unchanged in response to carnosol, hence suggesting that carnosol induces autophagy in Beclin-1-independent mode. To confirm that Beclin-1 is not needed for autophagy initiation, knocking down Beclin-1 using siRNA was done, they found that Beclin1-1 siRNA did not inhibit LC3II accumulation autophagy was successfully induced by carnosol [50].

This study showed that both cell death mechanisms, apoptosis, and autophagy, coexist in MDA-MB-231 cells upon the treatment with carnosol. Apoptosis was observed at higher concentrations of carnosol (50 and 100 μM), while autophagy was observed at a lower (non-cytotoxic) concentration (25 μM) and higher concentration as well. This indicates that MDA-MB-231 is treated with carnosol, firstly, it undergoes autophagy, and after more prolonged exposure to carnosol, which causes excessive cellular damage, this leads the cells to progress to apoptosis. To endorse this, a time-course experiment with carnosol treatment (100 μM) was used to check the expression of PARP (apoptosis marker) and LC3II (autophagy marker), LC3II was provoked at 3 h,

while cleaved PARP was started at 24 h [50]. This result clearly indicates that autophagy preceded apoptosis in carnosol-treated triple-negative MDA-MB-231 cells.

#### 1.3.3.3 Anticancer Effects of Carnosol through CYPs Enzyme Inhibition

In 2013, a study by Rodriguez and Potter evaluated the status of cytochrome P450-1A1 (CYP1A1) in estrogen receptor-positive (MCF-7) and triple negative (MDA-MB-231) breast cancer cell lines [62]. CYP1A1 is an enzyme that belongs to the cytochrome P450 superfamily and gets induced and expressed by the aryl hydrocarbon receptor (AhR), a transcription factor that gets activated via the binding to cytosolic ligands [84]. The enzyme CYP1A1 involves catalysis of several reactions in cholesterol, drugs, steroids, and metabolizing various procarcinogens to be active human carcinogens [85]. In addition to that, studies revealed that around 90% of breast cancer patients expressed CYP1A1 [86]. This raises the interest of CYP1A1 in breast cancer, hence exploring its activity in breast cancer progression and discovering its impacts on therapeutics will assist in developing and improving treatment strategies. Thus, Rodriguez and Potter decided to determine CYP1A1 biological properties and its role in the signal transduction of breast cancer cells, including triple-negative [62]. Firstly, to understand the biological role of CYP1A1 in both breast cancer cell lines MDA-MB-231 and MCF-7, they utilized siRNA to knock down CYP1A1 mRNA and protein. CYP1A1 silencing results in the impairment of cellular proliferation and survival, blocks the cell cycle at G0/G1, increases apoptosis, inhibits the ERK1/2 and AKT, and induces the AMPK pathway. After that, they sought to use carnosol as a pharmacological approach to reduce CYP1A1. Previous studies have shown that carnosol inhibits AhR and reduces CYP1A1 expression in premalignant tongue and prostate cancer [70,83]. Consistent with that, pharmacologic reduction of CYP1A1 levels by carnosol

also reduces proliferation, partly through the activation of the AMPK pathway [61]. Together these results suggest decreasing CYP1A1 levels using RNA silencing or carnosol might be a potential new method for triple-negative and estrogen-positive breast cancer treatment.

#### 1.3.3.4 The Synergetic Anticancer Effects of Carnosol in Combination Therapy

Despite the increasing interest in the anticancer effect of carnosol during cancer initiation and progression [56,84–90], few studies have proved the efficacy of using diterpenoids in monotherapy or when combined with other phytochemicals or chemotherapeutic agents [91–93]. To achieve this, Vergara and coworkers (2014) studied the antitumor activity of the dietary diterpene carnosol alone and in combination with other phytochemicals or with commonly used chemotherapeutic drugs using several human cancer cell lines including TNBC (HBL-100, MDA-231, and MDA-435) [62]. The results of the MTT assay revealed that carnosol alone decreases the cellular viability of TNBC and other cell lines in a dose- and time-dependent manner. Several researchers have demonstrated that a combination of phytochemicals can be more efficient in cancer prevention and treatment than single compounds [94,95]. For this reason, Vergara *et al*. treated the TNBC cell line (MDA-MB-231) with a combination therapy consisting of carnosol (50 μM) and curcumin (70 μM) for 4 hours and assessed their potential synergetic effects using western blotting. Combination therapy reduces Bcl-2, cyclin D1, and survivin while increasing p27 more efficiently than monotreatment. These results showed promising synergetic potential by reducing vitality, inducing apoptosis, and cause cell cycle blockade [62]. Overall, this study provided promising evidence on the synergetic anticancer effects of using carnosol in combination therapy to treat TNBC.

#### 1.3.3.5 Carnosol Stimulate ROS Production

A study by Wong and collaborators showed that ROS (reactive oxygen species) leads to the activation of apoptosis and beclin-1-independent autophagy in many cancer cell lines including the breast cancer MDA-MB-231 cells. In addition to that, ROS was found to activate ERK1/2 [96]. The ROS generation was evaluated with different concentrations of carnosol present using DCFDA fluorescence stain. ROS was found to be accumulated in carnosol treated MDA-MB-231 cells in a time- and concentration-dependent. Moreover, phosph-ERK1/2 and ∂H2AX (DNA damage marker) expression were elevated upon carnosol treatment [50]. Evidence in the literature showed that ROS trigger autophagy and apoptosis [97]. To investigate this, they treated MDA-MB-231 cells with carnosol and tiron (ROS scavenger), a decrease in DNA damage, LC3II accumulation, and cleaved PARP were observed [50]. This result demonstrates that carnosol exerts its anti-breast cancer effect through a ROS-dependent mechanism. Lastly, they found that carnosol also inhibits colony growth formation in TNBC cells.

## 1.4 Targeted Pathways in TNBC Progression in This Study

### 1.4.1 Cancer Migration, Invasion and Metastasis

Cancer metastasis is a process in which cancer cells spread to tissues and organs beyond where the primary tumor originated and forming new tumors (secondary and tertiary foci). Metastasis is the hallmark or cancer that being the key cause of cancer therapy failure and responsible for the greatest number of cancer mortality, and yet, it remains poorly understood [98]. Triple-negative breast tumors are an invasive type of cancer and usually characterized with poor prognosis and survival, relapse quickly,

and high incidence of metastasis. Treatment options for TNBC have been limited due to the absence of targetable hormone receptors and HER2 expression [99]. Although TNBC has a high response rate to chemotherapy at early treatment stages, it tends to relapse, and metastatic tumor develop. Once metastasis occurs, TNBC is incurable with an overall survival of only 10–13 months [100]. Hence, improving our understanding on the molecular mechanisms of TNBC metastasis might enhance the clinical management of this disease.

Metastasis is a complicated event that include a series of multiple steps. The metastasis cascade starts with detachment and migration of metastatic cells from the primary tumor, then, invading the basement membrane (BM) and extracellular matrix (ECM) surrounding the primary tumor (detachment, migration, and invasion). After that, invading the BM of the endothelium nearby blood and lymphatic vessels, by then, metastatic cells will intravasate the lymphatic system (intravasation) and will go through the circulation. Once metastatic cells reach the capillary endothelium of targeted organs, it adheres to the endothelium and invades the endothelial cell layer and the surrounding BM to extravasate to the targeted secondary tissue or organ (extravasation). Finally, metastatic cancer cells colonize and build blood vessels to enrich themselves (angiogenesis) and secondary tumor start growing [101–103].

#### 1.4.1.1 The Role of MMP-2 and MMP-9 in Cancer Metastasis

The regulation of metastasis includes various signaling pathways and is affected by the surrounding ECM components. One of the important steps in cancer metastasis is invasion, which involves the disassembly of extracellular matrix and its constituents by special enzymes, like matrix metalloproteinases (MMPs) [104]. MMPs are a family of zinc-dependent endoproteinases [105] and are known to play a key role in multiple

physiological processes, such as embryonic development, human reproduction and tissue remodeling, in addition to invasion and metastasis of cancer [106,107]. In tumor cells, the increased expression and activity of MMPs result in the degradation of ECM and BM, allowing tumor cells to invade other tissues and metastasis to distant organs [108]. Additionally, MMPs can modify cell adhesion molecules, and activate cytokines by proteolytic cleavage, resulting in the induction of epithelial-mesenchymal transition (EMT), a phenotypic alteration to enhance cell motility [109–111]. They also release soluble factors from the primary tumor site into the circulation, supporting establishing a metastatic niche in distant organs, subsequently helping tumor cells colonize [109]. Interestingly, MMPs not only produced by tumor cells, but also by stromal cell in the tumor microenvironment, as a consequent response to reciprocal paracrine interactions [112]. Several studies have reported an elevated MMPs protein expression in almost all cancer types and associated with poor prognosis [113].

There are several subtypes of MMPs that differ structurally and functionally within the MMPs family, among them the gelatinase MMP-2 and MMP-9, which are responsible for degrading gelatin, type IV and V collagens in BM and ECM through their proteolytic activity [114]. Some studies have revealed a link between the increased protein expression of MMP-2 and MMP-9 and the invasive capability of breast cancers has compared to a normal breast epithelium. In particular, breast cancer cells undergoing EMT have high expression of both MMP-2 and MMP-9 [115,116] , which have been implicated in breast cancer development and progression [117,118]. In addition, a study by Li *et al*. showed that TNBC cells (MDA-MB-231) had a significant increase in MMP-2 and MMP-9 mRNA and protein expression compared to human normal breast cell line (HS578Bst). These results correlate with the increase

in lymph node metastasis, tumor staging and poor prognosis [119]. Another report also showed that highly expressed MMP-2 and MMP-9 is also increasing the poor prognosis of triple-negative, with shorter overall survival and shorter progression-free survival [120]. Confirming previous studies, Sullu *et al*, have indicated that the high expression of MMP-9 is associated with poor prognostic factors, like tumor grade, distant metastasis [121]. Therefore, understanding MMP-9 and MMP-2 expression and activity regulation can help identify targeted therapies and guide TNBC prognosis.

#### 1.4.1.2 STAT3 Regulates the Expression of MMP-2 and MMP-9 in Cancer Metastasis

It has been reported that protein expression involved in the pathways of invasion (MMP-2 and MMP-9), EMT (twist and vimentin), angiogenesis (VEGF and HIFα), and proliferation and survival (cyclin D1, c-Myc, Bcl2, p53) are downstream of STAT3 transcriptional regulation. STAT3 (signal transducer and activator of transcription 3) is a member of the STAT family, and it is found constitutively active in all breast cancer subtypes, yet it is found highly expressed in TNBC and associated with its invasiveness [122–124]. The activation of STAT3 happens through the phosphorylation of its serine and tyrosine residues via signaling from upstream regulators such as cytokines and growth factors for receptor tyrosine kinases non-receptor tyrosine kinases, serine kinases and Gα-interacting vesicle-associated protein (GIV/Girdin)/guanine nucleotide exchange factor (GEF) [125,126]. Phosphorylated STAT3 induces homodimerization between two STAT3 molecules, then, formed homodimer translocate to the nucleus to bind to the consensus promoter sequence of its downstream target genes to initiate transcription [125].

Ma *et al.* have investigated the inhibition of STAT3 phosphorylation in breast cancer, which result in reduction of the expression of MMP-9, MMP-2 in addition to vasodilator-stimulated phosphoprotein (VASP) [127]. Additionally, Dai *et al.* revealed that posttranscriptional modification of STAT3 promote its phosphorylation which results in its activation, this ultimately cause an increase in MMP2 and MMP9 in the triple-negative MDA-MB-231[128]. The inhibition of the upstream regulator enzyme of STAT3 was found to reduce cell viability, migration, and invasion stimulated by MMP-2 and MMP-9 [128]. Further investigation of STAT3 stimulated MMPs expression in metastasis is crucial to find targeted therapies or enhance current treatment for metastatic TNBCs.

It is pertinent to state that the primary reason of death from breast cancer has been attributed to the distant metastasis. Oleandrin and odosroside are phytochemicals extracted from *Nerium oleander*. A study by Pongrakhananon has shown reduction in death among breast cancer patients who had treated with these compounds [129]. Another study confirmed that Oleandrin inhibits the activity of MMP9 and octamer binding transcription factor 3/4 (OCT3/4) through suppression of STAT3 at 50 and 100 nM used concentrations, respectively [130]. Moreover, Suh *et al.* revealed that treating breast cancer cell lines (MDA-MB-231 and MCF7) with resveratrol (a stilbenoid polyphenol found in grape skins and peanuts) was able to inhibit proliferation, migration, and invasion. In particular, resveratrol have found to downregulate the gene expression of Cyclin D1, c-Myc, MMP-9**,** MMP-2 and the protein expression of SOX2 as well as inhibit the activation of Akt and STAT3 in breast cancer cells [131].

The polyphenol carnosol have been experimented for its anticancer activity, and it has been found to suppress proliferation and migration in pancreatic cancer [132], brain cancer [133] and skin cancer [133]. These studies showed the therapeutic potential of using carnosol in cancer therapy, which make a promising target to treat the metastatic TNBC.

### 1.4.2 ROS Production Induces ER Stress and UPR in Cancer

The endoplasmic reticulum (ER) is a multifunctional organelle mainly responsible for the proteins folding and trafficking and maintaining other cellular functions. Changes in the protein-folding environment have led to the accumulation of unfolded or misfolded proteins in the ER lumen, profoundly affecting several cellular processes and causes ER stress [134]. The ER stress induces a collection of adaptive signaling pathways called an unfolded protein response (UPR) that involves the restoration of an efficient protein-folding environment and resolved misfolded protein [135]. However, if the ER stress is too severe to be rescued, UPR will trigger autophagy or apoptosis [136,137].

UPR is a highly regulated cascades system, and it involves three key sensors located on the ER membrane; protein kinase R-like ER kinase (PERK), transcription factor 6 (ATF6), and inositol requiring enzyme 1α (IRE1α) [135]. PERK is a kinase; upon ER stress, it phosphorylates the alpha subunit of the eukaryotic translation initiation factor-2 (eIF2α). Then, phosphorylated eIF2α rapidly attenuates protein translation and decreases the overload of proteins in the ER lumen. This action also promotes the expression of the UPR transcription factor ATF4, which triggers apoptosis through activating pro-apoptotic transcription factor CCAAT/enhancer-binding protein-

homologous protein (CHOP), which leads to apoptosis or induce cell cycle arrest [138,139]. CHOP can be induced by both cascades PERK/ATF4 and ATF6 [140–142]. Another UPR sensor is ATF6; under ER stress, it is transported to the Golgi apparatus and cleaved by specific proteases, which release its cytosolic fragment that function as a transcription factor. Activate ATF6 is known to regulate the expression of genes involved in the degradation of misfolded proteins [137]. The last UPR sensor is IRE1, a kinase and an endoribonuclease (RNase); upon its activation, it catalyzes the splicing of X-box binding protein 1(XBP1) mRNA. XBP1 is a transcription activator of essentials genes known to control protein folding, trafficking, phospho-lipid biosynthesis, and ER membrane expansion [143,144].

Accumulated evidence reported that UPR signaling cascades are activated in response to reactive oxygen species (ROS) accumulation or oxidative triggers [145]. Abnormal increases in ROS cause damage to cellular lipids, proteins, and DNA, resulting in irreversible oxidative damage, which ultimately leads to cell death and different pathological condition such as neurogenerative disease and cardiovascular disease [146]. On the other hand, several studies have suggested the beneficial effects of ROS generation on chemotherapy-induced cell death in cancer cells [147–149]. For example, bortezomib [150], apatinib (a TKI) [151], doxorubicin, inostamycin, vinblastin, xanthine oxidase–conjugated polymer, and camptothecin are chemotherapeutic agents that observed to induce apoptosis by increasing ROS production [152,153]. Since some tumors are more sensitive to ROS than normal cells [149], therefore, ROS generation could be an effective approach for killing tumor cells selectively without causing significant toxicity to normal cells [154,155]. Finding a new drug that stimulates ROS production

and ultimately leads to UPR activation is considered a goal for new effective therapeutic approaches in cancer treatment.

#### 1.4.2.1 The role of P38 in Regulating Cell death Upon ROS-induced ER Stress in Cancer

Over 33 years back, Ray and Sturgill have identified and characterized a new serine/threonine kinase signaling pathways family called MAPK (mitogen-activated protein kinase) [156,157]. MAPK signaling plays a crucial role in regulating various biological processes via different cellular mechanisms. MAPK signaling pathways getting activated via extracellular stimuli, such as oxidative stress, Gi-coupled receptor (GPCR), ultraviolet irradiation and genotoxic agents, inflammation, and other stimulants. Once activated or deactivated, MAPK will exert extracellular stimuli on a wide range of biological and cellular responses. These biological processes involved; cellular growth, proliferation, differentiation, migration, senescence, cell death [158,159] and others. MAPK is activated by dual phosphorylation on Thr and Tyr residues located in the kinase activation loop (T-loop) [160]. Their phosphorylation is done through a triple kinase module comprising a MAPK kinase kinase (MKKK) that phosphorylates and activates a MAPK kinase (MKK) which can trigger the terminal MAPK [161]. On the other hand, the inactivation of MAPK is through dephosphorylating its Thr and Tyr residues mediated by phosphatase [162]. In general, the cellular outcomes of MAPK cascades are dependent on different factors such as subcellular localization, amount and duration of MAPK activation, and the crosstalk with other signaling pathways inputs [163].

MAPK cascades are ubiquitous signal transduction pathways that regulate all aspects of life, and their alteration leads to human cancers and other diseases. There are three

prominent classical MAPK families in mammals: p38 kinase, C-Jun N-terminal kinase (JNK), and extracellular-regulated kinase (ERK1/2) [164]. MAPK is known to be highly involved in oncogenesis, tumor progression, and drug resistance [165]. P38 is a stress-activated MAPK, has pleiotropic effects on cancer, in which it has variable effects in different tumors. However, its outcomes are significant for the sensitivity to drug therapy [166]. For example, activated p38α was reported to induce cell cycle arrest and senescence [167–171]. Also, p38α can suppress breast, colon, liver, and lung tumor progression through ROS production and, consequently, mediate pro-apoptotic signals in these tumors [172]. On the other hand, p38 enhances lung metastasis of breast cancer through upregulating the expression of pro-metastatic genes [173]. Moreover, p38 was reported to enhance epithelial-to-mesenchymal transition and metastasis in other tumors [174,175]. Therefore, targeting the MAPK p38 in cancer therapy might have two-edged sword impacts.

### 1.4.3 The Role of Post-translational Modifications in Cancer Gene Expression

Eukaryotic DNA is tightly wrapped around a cluster of eight proteins named core histones to form the chromatin's basic units, which is nucleosomes. Post-translational modifications of histones, such as methylation, phosphorylation, ubiquitylation and acetylation, are essential components of the epigenome [176]. Acetylation is classified among the most abundant modifications involved in various key cellular processes such as cellular proliferation [177], cell-cycle progression, differentiation, apoptosis [178,179], dosage compensation, hormonal signaling [180], gene transcription, DNA damage repair, protein folding, autophagy and metabolism [181].

Acetylation of histones is a very well controlled process and it is one of the main epigenetic modifications that play a role in chromatin remodeling and dynamics with an impact on gene expression and transcriptional activities, and overall cellular homeostasis [182]. Histones or non-histones proteins acetylation being regulated by two classes of enzyme family members, working in an opposite mode: histone acetyltransferases (HATs) and histone deacetylases (HDACs) [183,184]. The dynamic and reversible acetylation of histone and non-histone proteins is a major epigenetic regulatory mechanism of genes transcription and deregulation of this process have been implicated in a wide variety of human diseases; for instance, developmental disorders [185–190], leukemia [191,192], pancreatic cancer [193], prostate cancer [194] and breast cancer [195], inflammation and immunity, and neurological and metabolic diseases such as diabetes [196,197]. Thus, proteins involved in the regulation of acetylation status considered as an attractive therapeutic target.

#### 1.4.3.1 The Role of P300 in Cancer Progression

Most of the canonical mammalian HATs are classified into three major families that have been well characterized: the GNAT, the MYST, and the P300/CBP families. The HATs p300/CBP were formerly discovered as E1A oncoprotein binding partners and cyclic AMP effectors, and then have addressed as global transcriptional coactivator [198]. In 1996, p300/CBP was described to possess HAT activity [199,200] and as a lysine acetyl-transferase is involved in regulating a wide range of biological processes [201]. Recent studies indicate that the HAT activity of p300/CBP have been associated with tumor development and progeression [202–205]. Deregulated HAT activity is particularly linked to cancer formation and progression [206,207], which is observed in several solid tumors, including prostate [201,208], colon [209,210] and breast [211,212] cancers.

Additionally, it have been shown that a functional p300 decrease the sensitivity to chemotherapy in estrogen-positive breast cancer, high recurrence and poor prognosis, mediate inflammation and tumor progression in triple negative breast cancer as well [213–216]. Therefore, HATs and HDACs are potential drug targets, and the restoration or modification of their activity could be beneficial for both the management and treatment of cancer [206,207].

Although there are increasing body of evidences suggests that p300 may be an important player in cancer [217]. However, its precise role is not clear and may depend on the physiologic background of the tumor [201]. Interestingly, evidence indicates that p300 can function both as a tumor suppressor and as an oncoprotein in cancer [217]. In fact, in invasive breast cancer, the high expression of p300 correlates with recurrence and poor patients' prognosis [216,218]. Moreover, p300 was reported to be involved in breast cancer progression by promoting cellular invasion and cellular survival [219]. Additionally, a functional p300 has been revealed to in association with a decrease in the sensitivity to chemotherapy in estrogen-positive breast cancer, high recurrence and poor prognosis, mediate inflammation and tumor progression in triple negative breast cancer as well [213–216]. Hence, targeting p300 in invasive breast cancer may be a promising approach for developing novel anticancer therapy.

Although studies on HDAC inhibitors have discovered highly potent compounds with rich clinical impact in cancer, the identification of HAT inhibitors has faced more challenges, and only a few potent ones have been identified [220,221]. These potent HAT inhibitors have been tested for their therapeutic potential in cancer [217,222,223] and other diseases [224–226].

PCAF (p300/CBP-associated factor), another histone acetyltransferase, and a component of several protein complexes was shown to play distinct roles in cancer, such as tumor suppressors, oncogenes, and transcription factors that control cell differentiation and cell cycle [227]. For instance, PCAF can acetylate NF-κB in response to inflammation and immunity [228], or it can acetylate p53 in response to DNA damage leading to cell cycle arrest [229]. Also, PCAF has been reported to function as a tumor repressor by promoting apoptosis in hepatocellular carcinoma [230] and inhibit the cell cycle in gastric cancer cells [231]. On the other hand, PCAF was found to promote tumorigenicity in lung adenocarcinoma via acetylating EZH2 [232] and enhances cellular proliferation of glioblastoma via Akt1 acetylation [233].

Recent investigations suggest that several dietary compounds have modified cancer risk epigenetically. Since post-translational modifications have been associated with the development and progression of some cancers, alteration of these events by dietary constituents provides a potential mechanism for their cancer-protective effects [220]. Various phytochemicals have been identified as modulators of the acetylation state of histones or affect the activities of HATs and/or HDACs [234]. Curcumin [235], anacardic acid [236], garcinol [237], epigallocatchechin 3-gallate [238], epigallocatechin-3-gallate (EGCG) [238], garcinol and isogarcinol [42,43] , veratric acid-derived [239] and plumbagin [240] have been showed to possess specific HAT inhibitor activity. Among these, curcumin was found to be the only known p300-specific natural inhibitor, both *in vitro* and *in vivo*, which is also cell-permeable [235,241]. Interestingly, curcumin's specific binding on p300/CBP led to a conformational change resulting in a decrease in the binding efficiency of histones H3, H4, and acetyl CoA [241].

Several screening researchers have identified natural product derivatives of potency as p300 HAT inhibitors; however, their selectivity and a detailed inhibition mechanism remain fully characterized. Carnosol is also a kind of polyphenol currently receiving increasing attention for its various health-promoting properties, including anti-breast cancer. Therefore, further investigation of the molecular mechanism of carnosol action on TNBCs, particularly on HATs activity, might reveal promising effects that result in enhancing current treatment.

# Chapter 2: Materials and Methods

## 2.1 Cell Culture and Chemicals

Human breast cancer cell lines, MDA-MB-231, MCF-7, T47D and Hs578T, were purchased from Cell Line Service (CLS)-GmbH and ATCC-USA. MDA-MB-231, Hs578T, and MCF-7 cell lines were cultured in DMEM medium and T47D in RPMI medium, the medium was supplemented with 10% fetal bovine serum (FBS) and 1% (100 µ/ml) penicillin/streptomycin, and the culturing condition was maintained at 37ºC, 5% $CO_2$ and 95% humidity. The reference of used cell lines, medium and its supplements, carnosol compound, apoptosis, autophagy and proteosome inhibitors, ROS scavenger, recombinant proteins, antibodies, and all other used reagents throughout the study are listed in the Appendix.

## 2.2 Wound Healing Assay

The migration capability of MDA-MB-231 and Hs578T cell lines was evaluated using Wound Healing Assay. Cells were cultured in 6-well plates overnight until it reaches confluency. The next day, a number of three wound lines were made in each well throughout the cells confluent monolayer using a sterile plastic pipette tip, 10 µl. Then, the cells were washed twice with PBS carefully, and cells were incubated at 37°C in a freshly prepared DMEM medium supplemented with FBS and antibiotic, and different concentration of carnosol were added accordingly. The wounds width was examined, and pictures were captured using inverted microscope at 100X magnification (Nikon Ti-U, Nikon) at indicated time points, 0 h and 5 h. The migration distance (d) of the cells was calculated as the following: d = (size of the wound at $t$ = 0 h - size of the wound at $t$ = 5 h).

### 2.3 Measurement of Cellular Viability

Cells were cultured in 12 well plates in a density of 40,000 cells/well and incubated overnight. The day after, cells were treated according to the plan and incubated for 24 h. Next, viable cells were collected and treated with the Muse Count and Viability Kit (Cat# MCH100102, Millipore, Hayward, CA, USA) reagents according to the manufacturer's protocols. The assessment of the number of viable cells was carried out using by Muse™ Cell Analyzer (Millipore).

Other assays were used to measure the cellular viability are through Cell Cytotoxicity Assay Kit (Cat# ab112118, Abcam, UK) and MTT Assay Kit (Cat# ab211091, Abcam, UK). Cells were seeded in 96-well plates at a density of 6,000 cells per well. After 24 h of culture, cells were treated with 0.2% (DMSO) or the indicated concentrations of carnosol and incubated for 24 h. After that, Kits reagents were applied according to the manufacturer's guideline. Viability was measured via monitoring the changes in the absorbance at 570 nm and 605 nm in a microplate reader, Platos R 496. The percentage of viability was calculated as following: % cell viability = 100 × (absorbance of treated sample / absorbance of control sample)

### 2.4 Matrigel Invasion Assays

BD Matrigel Invasion Chamber (Cat# 354480, 8 μm pore size; BD Biosciences, Bedfrord, MA, USA) was used to assess the invasiveness of MDA-MB-231 cells. According to manufacturer's instructions, in each well of the 24-well chamber plate, 5000 cells were placed in 0.5 mL of medium containing vehicle 0.2% (DMSO) or the indicated concentrations of carnosol, then cells were seeded into the upper chambers of the system; while the bottom wells in the system were filled with DMEM

supplemented with 10% FBS as a chemoattractant and then incubated at 37°C for 24 h. The cells that did not penetrate the matrigel were removed from the upper surface of the filter with a cotton swab. Cells that have migrated throughout the matrigel to the lower surface of the chamber were fixed with 4% formaldehyde and stained with DAPI. Fluorescence from DAPI stain was revealed 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 duplicates and repeated three times.

**2.5 Gelatin Zymography**

MDA-MB-231 ($2.5 \times 10^6$) cells were grown in serum-free medium containing vehicle 0.2% (DMSO) or the indicated concentrations of carnosol (25 and 50 μM) and incubated for 24 h. After 24 h of incubation, the medium was collected from culture and then concentrated. Total of 30 μg protein was resolved on non-reducing 10% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed for 1 h 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. Coomassie brilliant blue R-250 (Bio-Rad, CA, USA) staining was used with 0.5% to reveal gelatin-clear bands corresponding to the MMPs activity. The density of the bands were measured using ImageJ and band density was normalized to the non-specific band staining on the gel. The results showed represent two independent experiments.

## 2.6 Measurement of Matrix Metalloproteinase-9 by ELISA

Cells ($0.5 \times 10^6$) were seeded in 6-well plates in the presence of vehicle (0.2% DMSO) or carnosol (25 and 50 μM) for 24 h. Afterward, the conditioned medium was collected, and the levels of secreted MMP-9 were assessed using MMP9 Human ELISA kit (Abcam, Cambridge, UK) conferring to the manufacturer's protocol. The proteins present in the conditioned media were concentrated using the Amicon Ultra-0.5 protein purification and concentration column (Millipore). The protein levels of MMP-9 were normalized to the total protein level in each sample. The assays were performed three times in triplicates. Data are presented as mean values ± SEM.

## 2.7 Extraction of RNA and RT-PCR

MDA-MB-231 cells were treated with vehicle (0.2% DMSO) or carnosol (25 and 50 μM) for 24 h, then, the total RNA was extracted using TRIzol® Reagent (Cat# 15596026, Ambion, Life Technologies, Inc., USA) following the manufacturer's instructions. RNA expression of MMP-9 was determined by using the Qiagen OneStep RT-PCR kit (Cat# 210212, QIAGEN OneStep RT-PCR Kit, Qiagen, USA) according to manufacturer's guidelines. The amounts of RNA (500 ng) were equalized and used as templates in each reaction. The sequences of used primers are listed in Table 2.

Table 2: The sequences of the used RNA primers

| Primers sequences | | |
|---|---|---|
| MMP-9 | Forward | 5′- TTGACAGCGACAAGAAGTGG-3′ |
| | Reverse | 5′- CCCTCAGTGAAGCGGTACAT-3′ |
| MMP-2 | Forward | 5′-TCTCCTGACATTGACCTTGGC−3′ |
| | Reverse | 5′-CAAGGTGCTGGCTGAGTAGATC−3′ |
| GAPDH | Forward | 5′-GGCCTCCAAGGAGTAAGACC−3′ |
| | Reverse | 5′- AGGGGTCTACATGGCAACTG-3′ |

The PCR products were runed in 1.5% agarose gel and visualized via ethidium bromide staining. The results were representative from three independent experiments are shown.

### 2.8 Protein Extraction and Western Blotting Analysis

Cancer cell lines were cultured in a density of 2 $\times 10^6$ cell per 100 mm tissue culture dishe and incubated for 24 h. After that, treatment was applied for acquired times. Once the incubation time was done, cells were washed twice with ice-cold 1X PBS, scraped, collected by spinning down. The whole cell pellets were lysed in RIPA buffer (Cat# 89901, Pierce, Thermo Scientific, USA) containing phosphatase inhibitor cocktail; PhosSTOP EASTpack (Cat# 490684500, Roch, Germany) and cOmplete ULTRA (Cat# 5892970001, Roch, Germany) followed by 30 min incubation on ice. Later, cell lysates tubes were centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatants were collected. Total protein concentration was quantified through Pierce™ BCA Protein Assay Kit (Cat# 23227, Thermo Scientific, USA) and the background of the lysates were adjusted with lysis buffer. Aliquots of 20 µg of total cell lysate were loaded and resolved onto 6–15% SDS-PAGE. The electrophoresis running was on 1 X running buffer (30 g Tris-Base, Glycine 144 g and SDS 10 g), then proteins were transferred from the gels to Immobilon-P Transfer Membranes (Cat# IPVH00010, Millipore, Ireland) and wetted with 1X transfer Buffer (700 mL $dH_2O$, 200 mL absolute methanol and 100 mL Running Buffer) for 1 h and 15 min at 100 V at 4°C. Next, membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in 1X TBST (900 mL $H_2O$, 100 mL TBS and 0.01% Tween 20), 10 X TBS preparation as follow; Tis Base 24.2 g, NaCl 80 g and $H_2O$ 1 L. The membranes were immunoblotted with specific primary antibodies prepared in blocking buffer overnight

at 4°C and then with horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse or goat IgG. Immunoreactive protein bands were incubated with Pierce™ ECL Western Blotting Substrate (Cat# 32209, Thermo Scientific, USA) and SuperSignal™ West Femto Maximum Sensitivity Substrate (Cat# 34095, Thermo Scientific, USA) and chemiluminescence was detected using the LiCOR C-DiGit blot.

### 2.9 *In Vivo* Study Using Chick Embryo to Assess Tumor Growth and Metastasis of MDA-MB-231 Cells

The *in vivo* study was done using chick embryo to assess tumor growth and metastasis upon carnosol treatment. This experiment was performed by INOVOTION (Société: 811310127), La Tronche-France and reconfirmed again in our lab (Pr. Iratni's Lab) as previously described [242]. INOVOTION used fertilized White Leghorn eggs, obtained from the Société Française de Production Agricole (SFPA, St. Brieuc, France), and we used fertilized egg brought from local farms, all eggs were incubated at 38°C with 60% relative humidity for 10 days. As stated by the French legislation, no ethical approval is required for scientific experimentations using oviparous embryos (decree n° 2013–118, February 1, 2013; art. R-214–88). Animal studies were performed under animal experimentation permit N° 381029 and B3851610001 to Jean Viallet (INOVOTION). According to the Animal Research Ethics Committee in United Arab Emirates, ethical approval is needed (ERA-2018-5839, January 2019). Experiments were done at UAE University in E3 building.

At day E10, the upper chorioallantoic membrane (CAM) was dropped by drilling a small rounded hole (1 $cm^2$) though the eggshell into the air sac and a window was created in the eggshell above the CAM. Cultured MDA-MB-231-GFP were trypsinized, then, neutralized with complete medium and suspended in serum free

DMEM. A 50 µl containing 1 x $10^6$ MDA-MB-231-GFP cells was added onto the upper CAM of each egg. Eggs were split randomly onto 4 groups of 15 eggs (to get sufficient surviving embryos at the end of the experiments). One day later at E11, tumors began to be visible. Tumors were treated every 2 days (E11, E13, E15, E17), by dropping 100 µl of either carnosol (50 or 100 µM), colchicine (2 µM) as a positive control, or vehicle (0.02 % DMSO) prepared in 1X PBS onto the tumor. At E19 the upper portion of the CAM which include tumor was removed, transferred in 1X PBS and the tumors were carefully cut away from normal CAM tissue and weighed. In parallel, a 1 $cm^2$ portion of the lower CAM was collected to calculate 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 1X PBS and compressed between a hollow glass slide and a thick coverslip. In order to count the nodule, Leica Macrofluo fluorescent microscope equipped with GFP filter was used to scan the previously prepared lower CAM samples. At the end, chick embryos were sacrificed by decapitation.

### 2.10 Assessing Histone Acetyltransferase Activity Using Colorimetric Assay and *in Vitro* Experiment

HAT activity was measured using the commercially available colorimetric HAT activity assay kits such as Hitson Acetyltransferase Activity Assay Kit (Cat# ab65352, Abcam, UK) and HAT Activity Assay Kit (Cat# ALX-850-326-KI01, Enzo, US). Briefly, 50 µg of HeLa cell nuclear extract (Santa Cruz Biotechnology) was incubated, with or without carnosol, in HAT assay buffer according to the manufacturer's instructions. Absorbance was measured at 450 nm using the microplate reader, Platos

R 496. Experiments were done in triplicate and repeated in triplicates. Data are represented as mean values ± SEM.

For the *in vitro* analysis 100 ng of recombinant HATs (p300, PCAF or GCN5) was incubated in the presence of a HAT assay buffer (50 mM tris pH8.0, Glycerol 10%, 0.1 mM EDTA, 1 mM dithiotheithol, 1 mM PMSF), 400 nM trichostatin A, 20 µM Acetyl-CoA in the presence of DMSO, as control, or carnosol for 1 h at 30°C. The reaction was stopped by the addition of SDS-loading buffer (4X). HAT activity was determined by Western Blotting using specific antibodies specific for acetylated histones. Experiments were carried out in triplicate and repeated three times. Data are represented as mean values ± SEM.

**2.11 Statistical Data Analysis**

The statistical analysis in the study was carried out through SPSS version 21 for PC (IBM Corp.) and Microsoft Office Excel version 16.52 for Mac (21080801). Data were reported as means ± SEM Differences between groups were analyzed using a Student's I-test for paired or unpaired values. The significancy for all data was <0.05 for the P-value and taking into consideration that all experiments were repeated at least three times and sometimes more.

# Chapter 3: Results and Discussion

## 3.1 Carnosol Inhibits Migration, Metastasis, and Tumor Growth of Breast Cancer via a ROS-Dependent Proteasome Degradation of STAT3

### 3.1.1 Results

#### 3.1.1.1 Carnosol Inhibits Migration of TNBC Cell Lines

TNBC is one of the aggressive diseases known for its high prevalence due to its migration capacity and metastasis [243]. Cell migration capability is an initial and critical step in the progression of cancer invasion and metastasis [244]. Therefore, the effect of carnosol on the migratory capacity of TNBC cell lines, MDA-MB-231, and Hs578T cells was analyzed using a wound-healing assay. The results showed that carnosol significantly inhibited cellular migration of MDA-MB-231 (Figure 2A and 2B) and Hs578T cells (Figures 2D and 2E).

To rule out the possibility that the inhibition of cell migration is due to drug-induced cell death, treated and non-treated (control) MDA-MB-231 cells were collected at the end of the experiment by trypsinization, and the number of viable cells was quantified. As is shown in Figure 2C, the number of viable cells did not decrease in carnosol-treated wells when compared to control.

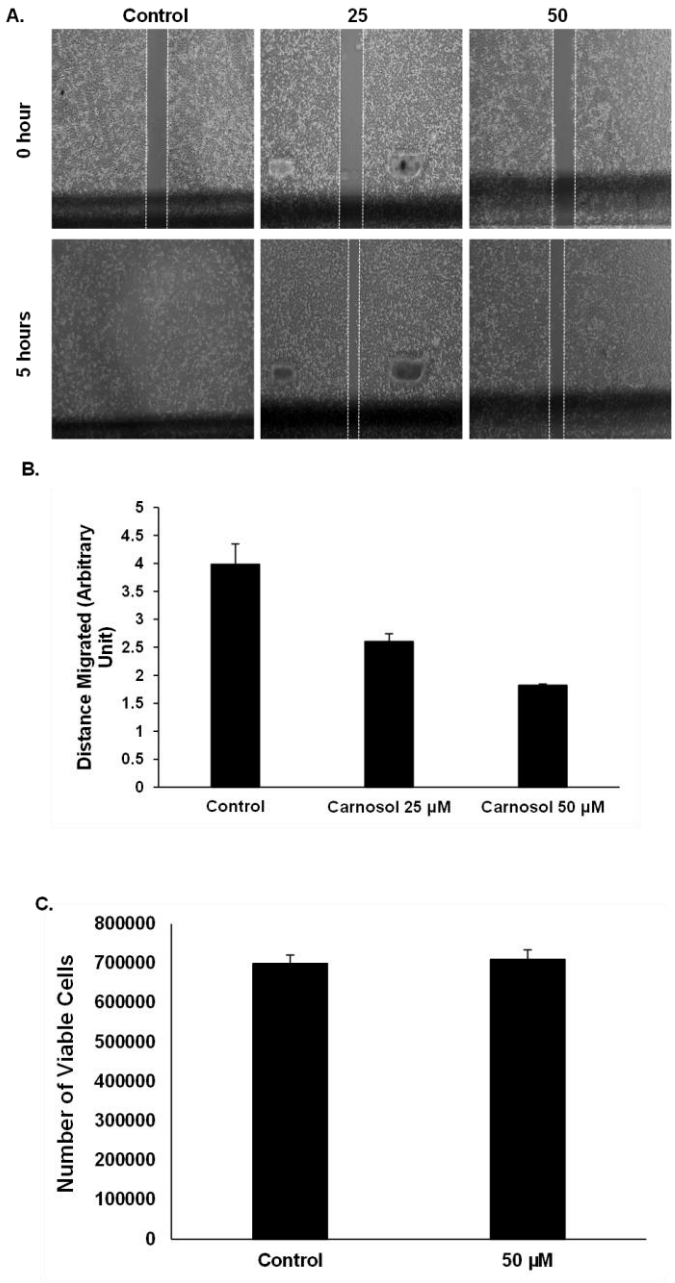


Figure 2: Carnosol hinders the migration of MDA-MB-231 and Hs578T cells.

(A) MDA-MB-231 cells were cultured till reaching confluency, a wound was introduced by scratching with a pipette tip and the cells were incubated in DMEM without and with the indicated concentrations of carnosol. The wound was examined with an inverted microscope 40X magnification and photographed. (B) Quantitative assessment of the wound healing assay. Values represent the mean ± SEM (n = 3) distance (arbitrary unit) that the cells have migrated 5 h post-treatment (*$p < 0.05$). (C) Cellular viability of carnosol-treated MDA-MB-231 cells measured after 5 h post-treatment. Results shows that no significant difference in number of viable cells between carnosol-treated and control cells. (D) Hs578T cells were cultured till reaching confluency, a wound was introduced by scratching with a pipette tip and the cells were incubated in DMEM without and with the indicated concentrations of carnosol. The wound was examined with an inverted microscope 40X magnification and photographed. (E) Quantitative assessment of the wound healing assay. Values represent the mean ± SEM (n = 2) distance (arbitrary unit) that the cells have migrated 5 h post-treatment (**$p<0.005$).

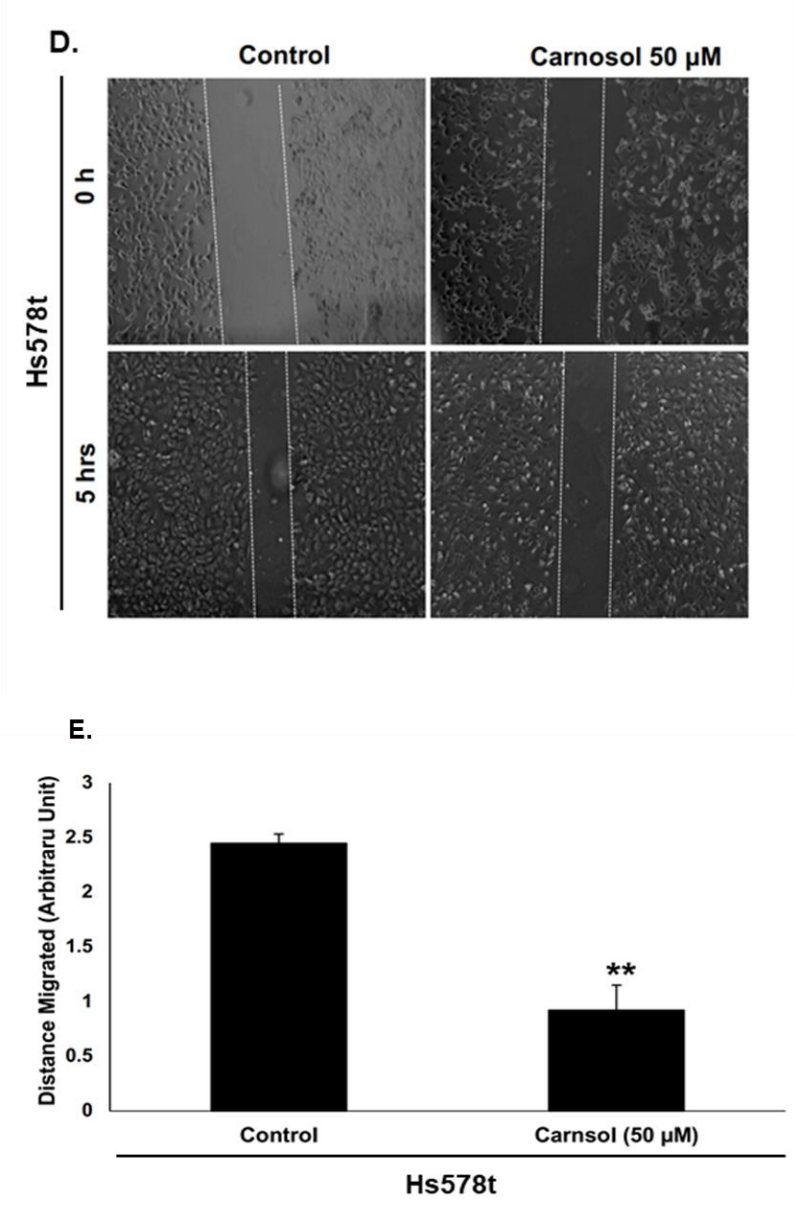


Figure 2: Carnosol hinders the migration of MDA-MB-231 and Hs578T cells (Continued).

(A) MDA-MB-231 cells were cultured till reaching confluency, a wound was introduced by scratching with a pipette tip and the cells were incubated in DMEM without and with the indicated concentrations of carnosol. The wound was examined with an inverted microscope 40X magnification and photographed. (B) Quantitative assessment of the wound healing assay. Values represent the mean ± SEM (n = 3) distance (arbitrary unit) that the cells have migrated 5 h post-treatment (*$p < 0.05$). (C) Cellular viability of carnosol-treated MDA-MB-231 cells measured after 5 h post-treatment. Results shows that no significant difference in number of viable cells between carnosol-treated and control cells. (D) Hs578T cells were cultured till reaching confluency, a wound was introduced by scratching with a pipette tip and the cells were incubated in DMEM without and with the indicated concentrations of carnosol. The wound was examined with an inverted microscope 40X magnification and photographed. (E) Quantitative assessment of the wound healing assay. Values represent the mean ± SEM (n = 2) distance (arbitrary unit) that the cells have migrated 5 h post-treatment (**$p<0.005$).

### 3.1.1.2 Carnosol Decreases Invasive Capacity of MDA-MB-231 Cells

Cancer cells invasion is another critical step in metastasis that involves breaching tissue barriers by cancer cells, which subsequently lead to the infiltration of these cells into surrounding tissue [245]. One of the hallmarks of metastasis breast cancer is increasing its invasion capacity [246]. Since carnosol had shown to suppress the migration ability of TNBC cell lines, towered this, assessing the effect of carnosol on the invasive potential of MDA-MB-231 cells using matrigel Invasion assay was done. The assay revealed that the invasive ability of MDA-MB-231 was significantly reduced by carnosol compared with the control cells (Figure 3A). Then, a quantification of invaded MDA-MB-231 cells into the matrigel has shown that upon carnosol treatment, the number of cells that invaded the matrigel coated membrane has significantly reduced by 60%. Of note, LY294002, a drug used as a positive control, strongly suppressed the invasion of MDA-MB-231 as well (Figure 3B). These results indicate that carnosol has a significant suppressive capacity on the invasion of TNBC cells.

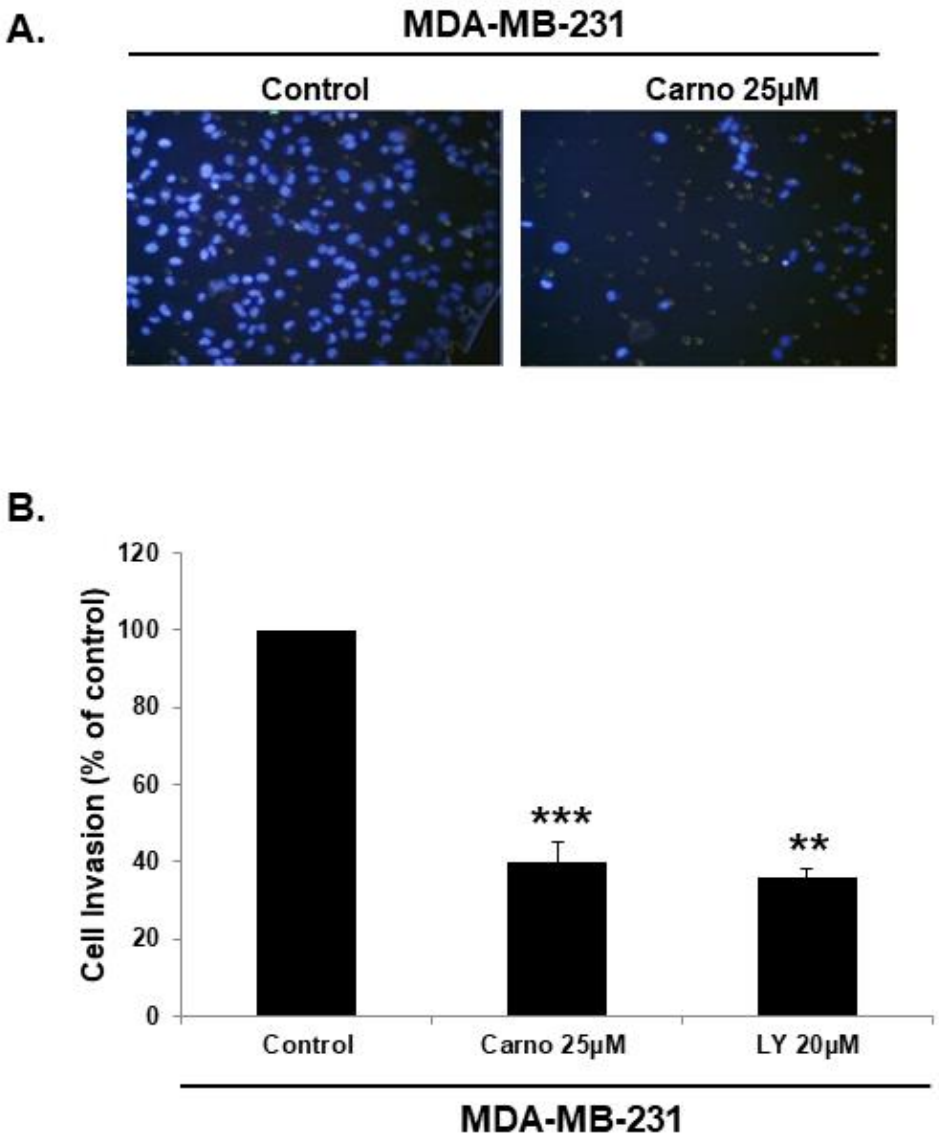


Figure 3: Carnosol constrains the invasion ability of MDA-MB-231 cells.

(A) MDA-MB-231 cells were incubated for 24 h with 25 µM of carnosol or without (a vehicle of 0.2% DMSO) and LY294002 (20 µM). MDA-MB-231 Cells that were able to invade into the matrigel were scored as described in Materials and Methods. (B) Quantitative analysis of invaded MDA-MB-231 into the matrigel. Values represent means ± SEM, n = 3 (**$p < 0.005$ and ***$p < 0.001$).

### 3.1.1.3 Carnosol Downregulates the Expression and the Activity of MMP-9 in MDA-MB-231 Breast Cancer Cells

It is well known that MMP enzymes involve in the disassembly and degradation of ECM and its constituents to facilitate cancer cell migration and invasion [104]. Several studies have revealed a link between the increased protein expression of MMP-9 and MMP-2 and the invasiveness of breast cancers [115]. Particularly, breast cancer cells undergoing EMT have shown elevated expression of both MMP-9 and MMP-2 [116], which have been implicated in breast cancer development and metastasis [118].

This draws us to analyze the effect of carnosol on the activity of MMP-9 and MMP-2 in breast cancer cells. Using gelatin zymography assay, carnosol significantly reduced the activity of MMP-9 in a concentration-dependent manner in MDA-MB-231 cells (Figure 4A). On the other hand, the activity of MMP-2 was not affected by carnosol (Figure 4D). Next, to test whether carnosol inhibits breast cancer cell invasion by affecting the expression of MMP-9, evaluating the expression level of MMP-9 in the conditioned media was performed. As shown in Figure 4B, levels of secreted MMP-9 were significantly reduced in carnosol-treated MDA-MB-231 cells. Further, RT-PCR analysis was achieved to evaluate the mRNA expression of MMP-9. The results revealed that MMP-9 expression was down-regulated in carnosol-treated cells (Figure 4C). At the same time, the level of MMP-2 transcripts in carnosol-treated cells was not affected in comparison to control cells (Figure 4E). Altogether, these data clearly indicate that carnosol suppresses the expression and activity of MMP-9 activity.

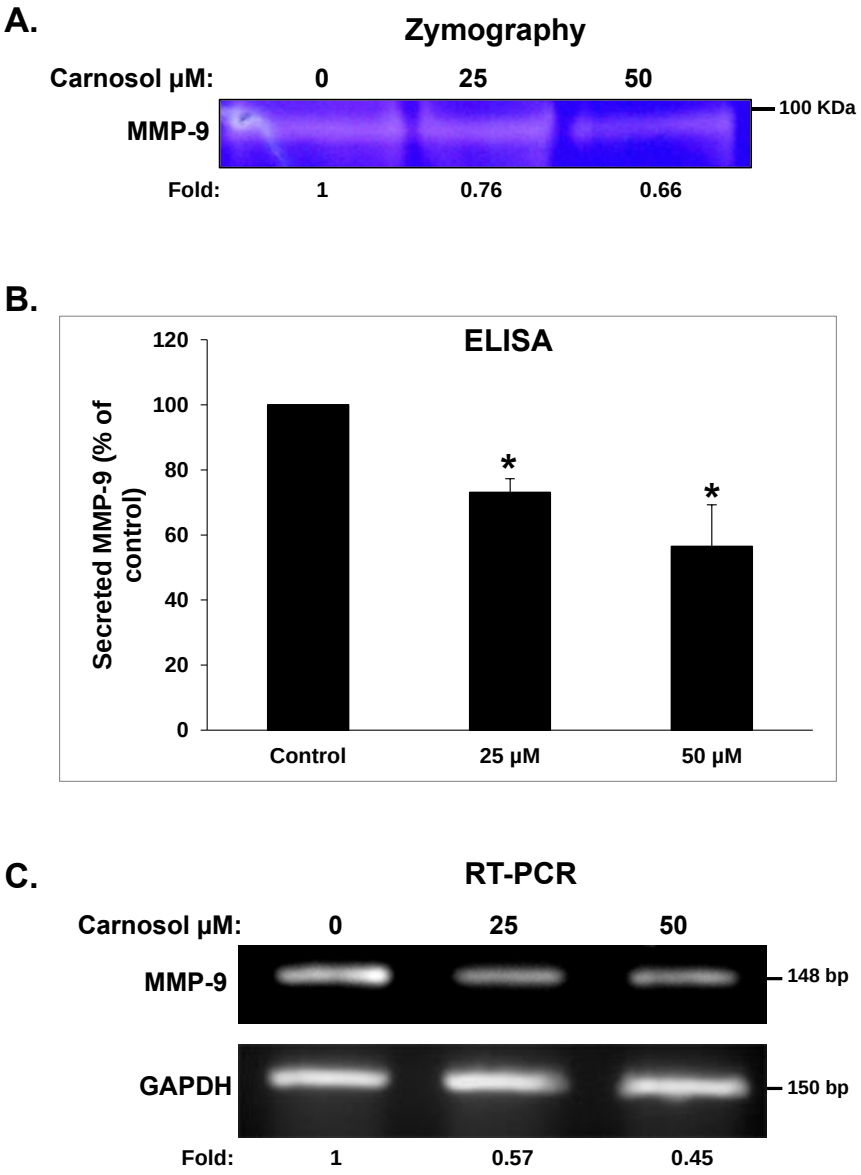


Figure 4: Carnosol downregulates MMP-9 activity and expression in MDA-MB-231 cells.

(A) The activity of MMP-9 in carnosol-treated MDA-MB-231 cells. Cells were treated with 25 μM and 50 μM of carnosol for 24 h and then to measure the activity of MMP-9 samples were subjected to gelatin zymography (B) Effects of carnosol on the secretion of MMP-9 in conditioned media. The levels of secreted MMP-9 were evaluated using immunoassay kits as described in Materials and Methods. Values represent the mean three samples ± SEM. (C) Effects of carnosol on the expression level of MMP-9 mRNA. Cells were treated with vehicle (0.2% DMSO) or carnosol (25 μM and 50 μM) for 24 h and the subjected to RT-PCR to examine the mRNA level of MMP-9 transcript. GAPDH was used as an internal control. Values represent the mean ± SEM (n = 3) (*$p < 0.05$). (D) Carnosol has no effect on the expression and activity of MMP-2, a measurement of the activity of MMP-2 in carnosol-treated MDA-MB-231 cells. Cells were treated with 25 μM and 50 μM carnosol for 24 hours and then subjected to gelatin zymography, to measure the activity of MMP-2. (E) Effects of carnosol on the expression level of MMP-2 mRNA. Cells were treated with vehicle (0.2% DMSO) or carnosol (25 μM and 50 μM) for 24 h and subjected to RT-PCR to reveal the mRNA level of MM2-9 transcript. GAPDH was used as an internal control.

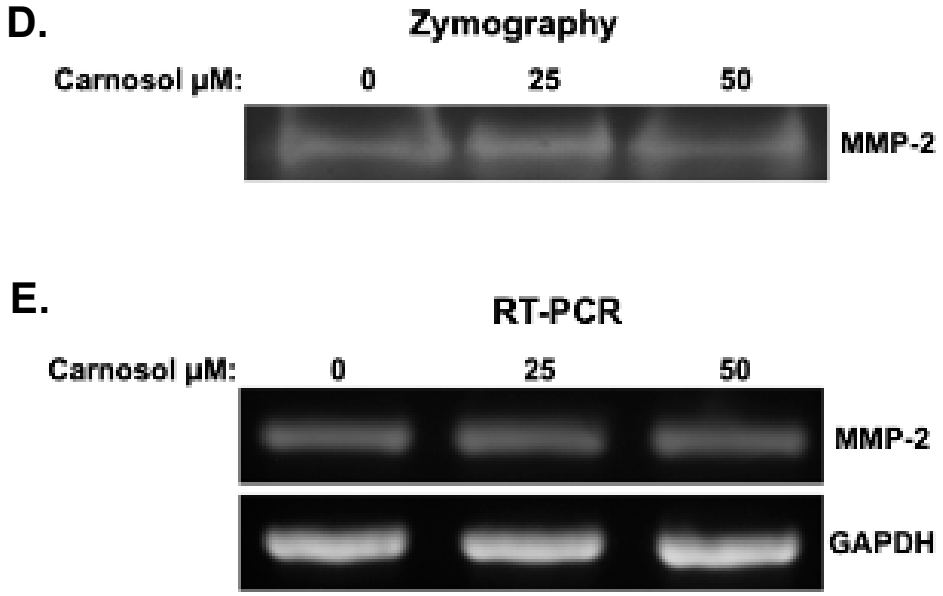


Figure 4: Carnosol downregulates MMP-9 activity and expression in MDA-MB-231 cells (Continued).

(A) The activity of MMP-9 in carnosol-treated MDA-MB-231 cells. Cells were treated with 25 μM and 50 μM of carnosol for 24 h and then to measure the activity of MMP-9 samples were subjected to gelatin zymography (B) Effects of carnosol on the secretion of MMP-9 in conditioned media. The levels of secreted MMP-9 were evaluated using immunoassay kits as described in Materials and Methods. Values represent the mean three samples ± SEM. (C) Effects of carnosol on the expression level of MMP-9 mRNA. Cells were treated with vehicle (0.2% DMSO) or carnosol (25 μM and 50 μM) for 24 h and the subjected to RT-PCR to examine the mRNA level of MMP-9 transcript. GAPDH was used as an internal control. Values represent the mean ± SEM (n = 3) (*$p < 0.05$). (D) Carnosol has no effect on the expression and activity of MMP-2, a measurement of the activity of MMP-2 in carnosol-treated MDA-MB-231 cells. Cells were treated with 25 μM and 50 μM carnosol for 24 hours and then subjected to gelatin zymography, to measure the activity of MMP-2. (E) Effects of carnosol on the expression level of MMP-2 mRNA. Cells were treated with vehicle (0.2% DMSO) or carnosol (25 μM and 50 μM) for 24 h and subjected to RT-PCR to reveal the mRNA level of MM2-9 transcript. GAPDH was used as an internal control.

#### 3.1.1.4 Carnosol Inhibits STAT3 Pathway by Downregulating Both Active and Total STAT3 in Breast Cancer Cell Lines

STAT3 plays a crucial role in signaling pathways that promote cellular proliferation, tumor angiogenesis, invasion, and migration. The active form of STAT3 is constitutively and aberrantly activated in many types of cancers, including TNBC, where it promotes tumorigenesis [247]. Thus, STAT3 is widely recognized as a promising therapeutic target in cancer treatment.

Whether carnosol affects constitutively active STAT3 in MDA-MB-231 cells was investigated. Toward this, cells were treated with carnosol (25, 50, and 100 μM) for 24 h. Carnosol was found to dramatically downregulated the level of phosphoSTAT3 in a concentration-dependent manner (Figure 5A, upper panel). Strikingly and unexpectedly, cells treated with carnosol showed a marked reduction in the level of total STAT3 (Figure 5A, lower panel). This finding prompted us to investigate whether carnosol inhibits the STAT3 pathway in other breast cancer cell lines by downregulating the level of total STAT3. In this direction, the level of total STAT3 was assessed in three other breast cancer cell lines (Hs578T, MCF-7, and T74D) cell line. We found that carnosol also caused a marked decrease in the level of total STAT3 in these three cell lines as well (Figure 5B). Interestingly, and in agreement with previous work [248], we found that the expression of STAT3 was not affected in HCT116 colorectal cancer cells after carnosol treatment (Figure 5C). In summary, our data strongly suggest that carnosol inhibits the STAT3 pathway through a breast cancer-specific mechanism.

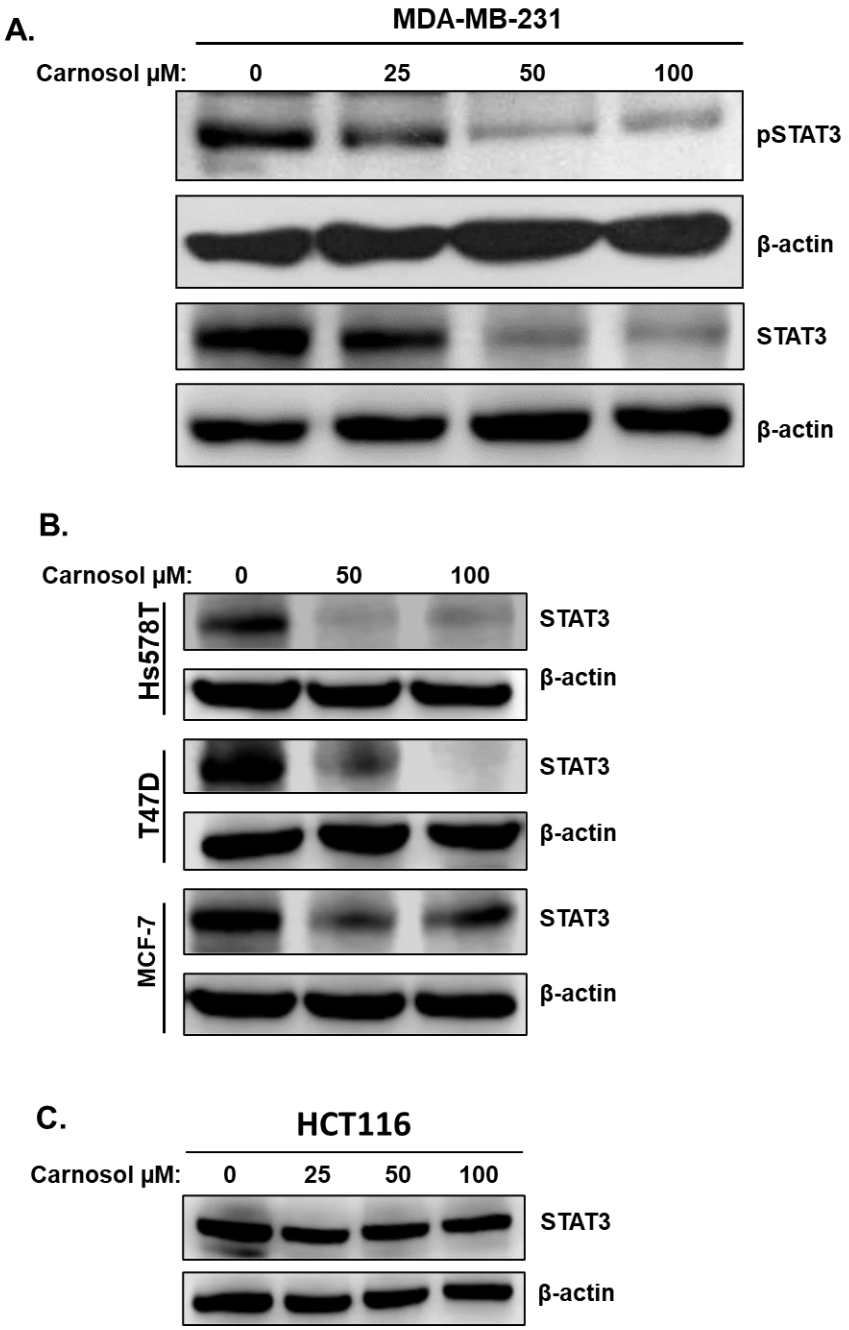


Figure 5: Carnosol inhibits STAT3 signaling pathway.

(A) Phospho-STAT3 and STAT3 protein were decreased in a concentration-dependent manner in carnosol-treated MDA-MB-231 cells. Cells were treated with vehicle (0.2% DMSO) or indicated concentrations of carnosol (25 μM, 50 μM and 100 μM) for 24 h, then whole-cell extracts were subjected to Western Blotting analysis for the phosphorylated STAT3, total STAT3form and for β-actin (loading control). (B) Carnosol downregulated the level of STAT3 protein in Hs578T, MCF-7, and T47D breast cancer cell lines. Whole cell lysates from different breast cancer cell lines treated with the indicated concentrations of carnosol were resolved on 8% SDS-PAGE and analyzed by Western blotting for STAT3 protein. (C) Western blotting analysis of STAT3 protein level in HCT116 colorectal cancer cells. Western blotting was implemented as described above.

### 3.1.1.5 Carnosol Targets STAT3 to Proteasome-Mediated Degradation

To investigate the mechanism by which carnosol downregulates the level of total STAT3 in breast cancer cells, the level of STAT3 protein in control and carnosol-treated MDA-MB-231 cells was examined by Western Blotting. Al Dhaheri *et al*. have previously shown that carnosol triggered autophagy in breast cancer cells [50]. Therefore, an assessment of whether STAT3 was degraded by autophagy was done. The result showed that blockade of early-stage autophagy (autophagosome formation) by 3-Methyladenine (3-MA) and late-stage autophagy (autophagolysosome formation) by Chloroquine (CQ) did not restore the level of STAT3 protein (Figure 6A). This suggests that the decrease of STAT3 proteins in response to carnosol is autophagy-independent.

To determine whether the proteasomal activity is involved in the carnosol-mediated decrease of STAT3 protein, a pre-treatment for MDA-MB-231 cells with or without the proteasome inhibitors carbobenzoxy-Leu-Leu-leucinal (MG-132) and bortezomib was applied for 1 h and then with carnosol. As shown in Figure 6B, both inhibitors efficiently restored STAT3 to a level comparable to that in control untreated cells. This is a clear indication that carnosol targets STAT3 to proteasome degradation. In addition, the results revealed that the restoration of STAT3 protein did not restore the level of phosphorylated STAT3 (Figure 6B). Moreover, an examination of the effect of proteasome inhibitor on STAT3 in another TNBC cell line, namely Hs578T was carried out. As shown in Figure 6C, bortezomib restored the level of STAT3 protein. Altogether, these data suggest that carnosol might exert its effect, at least partly, through inhibition of the STAT3 signaling pathway by targeting STAT3 to proteasome degradation in breast cancer.

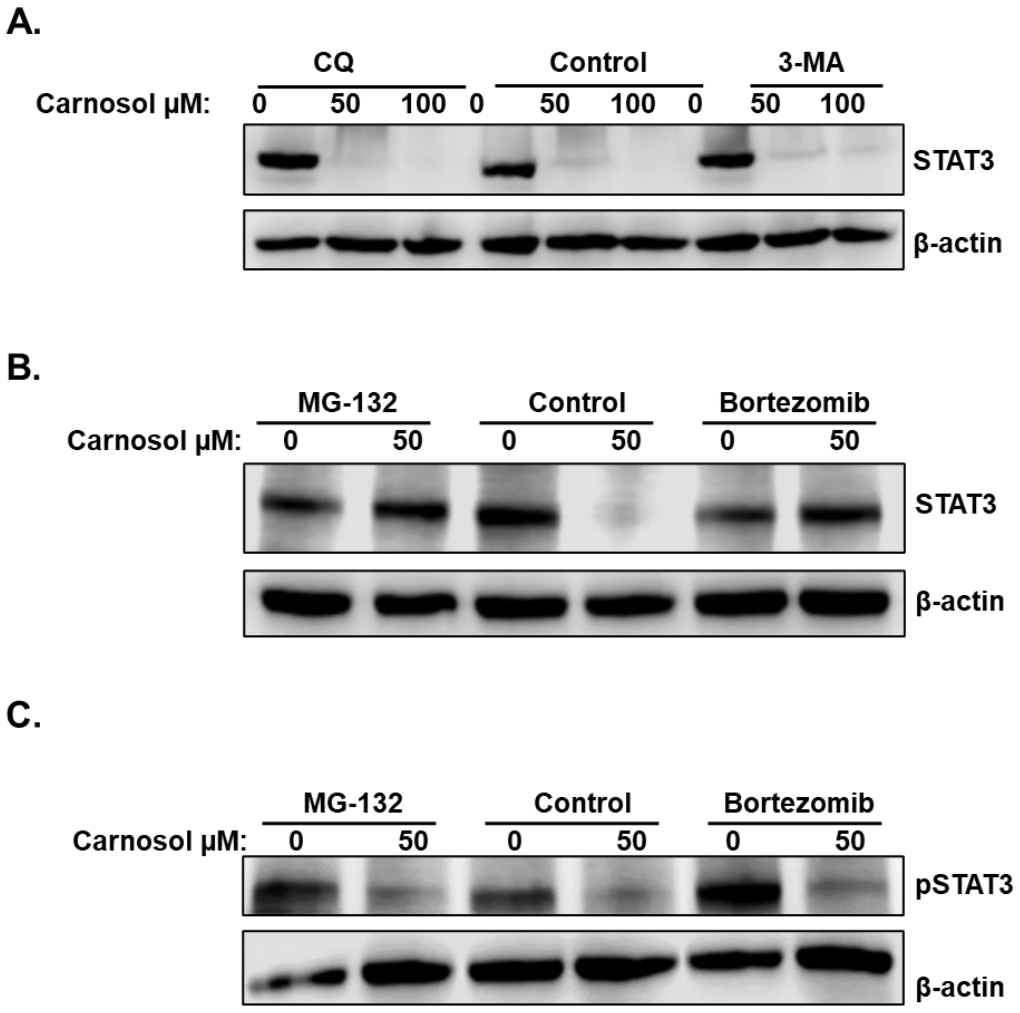


Figure 6: Proteasome-dependent degradation of STA3 protein in carnosol-treated MDA-MB-231 and Hs578t cells.

(A) Inhibitors of autophagy didn't restore STAT3 protein expression level. Western blot experiment of STAT3 protein expression level in MDA-MB-231 cells pretreated with the autophagy inhibitors; CQ and 3-MA. The cells firstly were pretreated with or without 3-MA (50 mM) and CQ (50 µM) for 1 h and, followed by the addition of carnosol, and cells were further incubated for 24 h. (B) Inhibitors of the proteasomes (MG-132 and Bortezomib) returned STAT3 protein to a level comparable to control cells. MDA-MB-231 were pre-treated for 1 h with or without autophagy inhibitors; MG-132 (15 µM) or Bortezomib (25 nM), followed by the addition of carnosol, and cells were further incubated for 24 h. Whole cells lysate were resolved on 8% SDS-PAGE and examined by Western blotting for total STAT3 and phospho-STAT3 protein. (C) Carnosol induces proteasome-dependent degradation of STAT3 in Hs578T. Inhibitors of the proteasomes (Bortezomib) rescued STAT3 protein from proteasome degradation. Hs578T cells were pre-treated for 1 hour with or without Bortezomib (25 nM) before treatment with carnosol at the indicated concentrations. Whole cells lysates were resolved on 8% SDS-PAGE and evaluated by Western blot for STAT3 protein.

### 3.1.1.6 Carnosol Promotes Proteasome Degradation of STAT3 Through a ROS-Dependent Mechanism

Another study besides Al Dhaheri *et al*. has previously shown that carnosol induced ROS in breast [50] and colon [248] cancer cells. This prompted us to test whether ROS contributes to STAT3 degradation. MDA-MB-231 cells were pre-treated with the ROS scavenger, N-Acetyl Cysteine (NAC), for 1 h, and this was followed by treatment with or without carnosol. As shown in Figure 7, blockade of ROS efficiently rescued STAT3 from proteasomal degradation. This result demonstrates that carnosol targets STAT3 to proteasome degradation through a ROS-dependent mechanism.

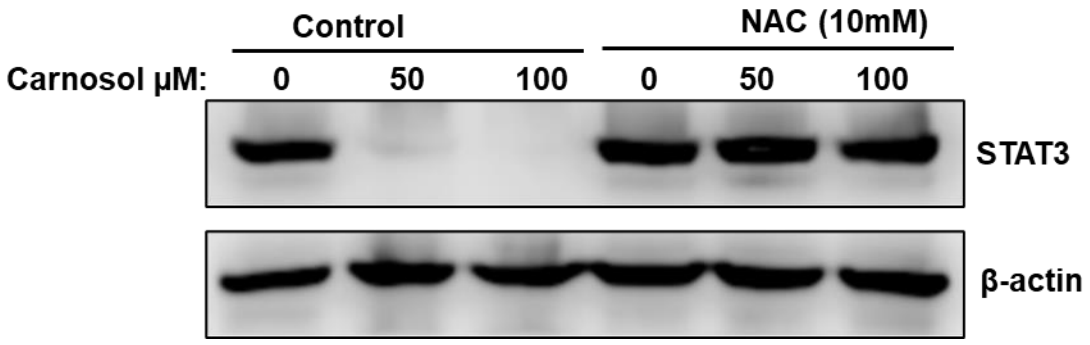


Figure 7: STAT3 degradation in a ROS-dependent manner.

MDA-MB-231 cells were pre-treated for 1 h with the ROS scavenger, NAC, then carnosol (50 and 100 μM) was added. Whole cells lysate was resolved on 8% SDS-PAGE and revealed by Western blotting for STAT3 protein.

### 3.1.1.7 Carnosol Inhibits Tumor Growth and Metastasis of MDA-MB-231 Cells

Al Dhaheri *et al*. showed that carnosol significantly decreased the viability and colony growth of MDA-MB-231 cells [50]. To further confirm these *in vitro* activities of carnosol, *in vivo* experiment was carried out to investigate the effect of carnosol on tumor growth using the chick embryo model. The highly invasive MDA-MB-231 cells

were implanted on the chorioallantoic membrane (CAM), and then the formed tumors were treated with vehicle (DMSO), colchicine (a positive control), or carnosol. At E 19, the tumors were recovered from the upper CAM and weighed each. The results demonstrated that carnosol concentrations of 50 and 100 μM significantly inhibited tumor growth by 65 and 75%, respectively, compared with the DMSO treatment (Figures 8A, B). Colchicine treatment (2 μM) reduced tumor growth by 65%. Furthermore, the ability of carnosol to inhibit metastasis was examined *in vivo*. To this end, the number of nodules was counted in the lower CAM in DMSO-, colchicine- and carnosol-treated tumors. An average of 6 nodules in the lower CAM of vehicle-treated chick embryos were found, while an average of only 0.7 nodules was counted in carnosol-treated embryos (Figure 8C). Taken together, these outcomes demonstrate that carnosol strongly inhibits breast tumor growth and metastasis *in vivo*. Of note, carnosol showed no cytotoxicity, as there was no difference in the number of surviving embryos in control and carnosol-treated embryos.

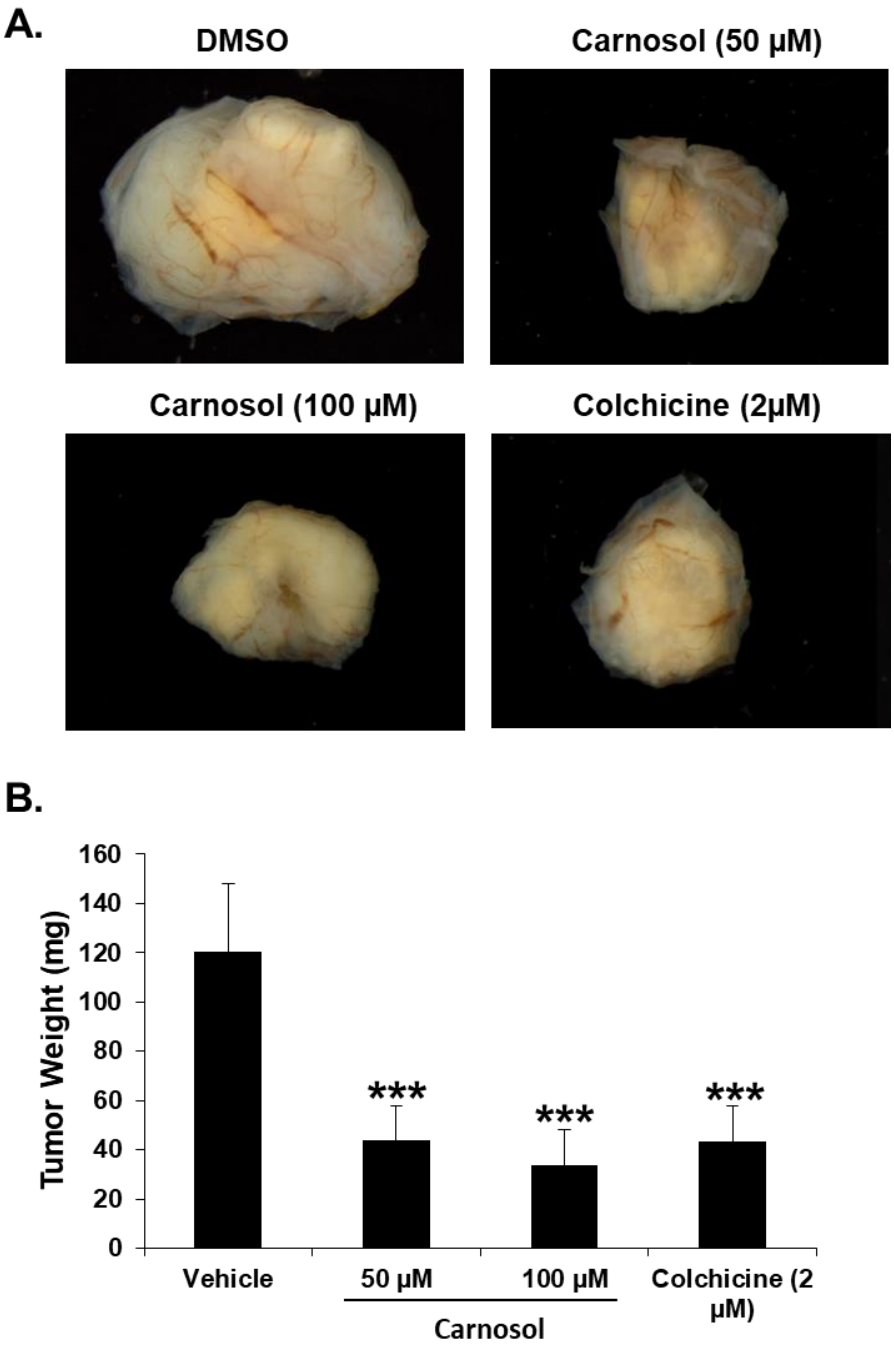


Figure 8: Antimetastatic and antitumor growth activity of carnosol on breast tumor in chick embryo chorioallantoic membrane assay.

(A) MDA-MB-231 ($1\times10^6$) cells were grafted on the CAM of 10-day chick embryo (E10). Every 48 h Tumors were treated with carnosol (50 µM and 100 µM) or 0.2% DMSO (vehicle) or colchicine (2 µM) as described in Materials and Methods. Later, tumors were collected and weighted at day 19 (E19). (B) Weight measurements of tumors in vehicle-, colchicine- and carnosol-treated chick embryo. (C) Antimetastatic effect of carnosol assessed by counting the nodules observed in the lower CAM of chick embryo treated with vehicle, colchicine or indicated concentrations of carnosol. Columns represents mean; bars represent SEM (***$p< 0.001$).

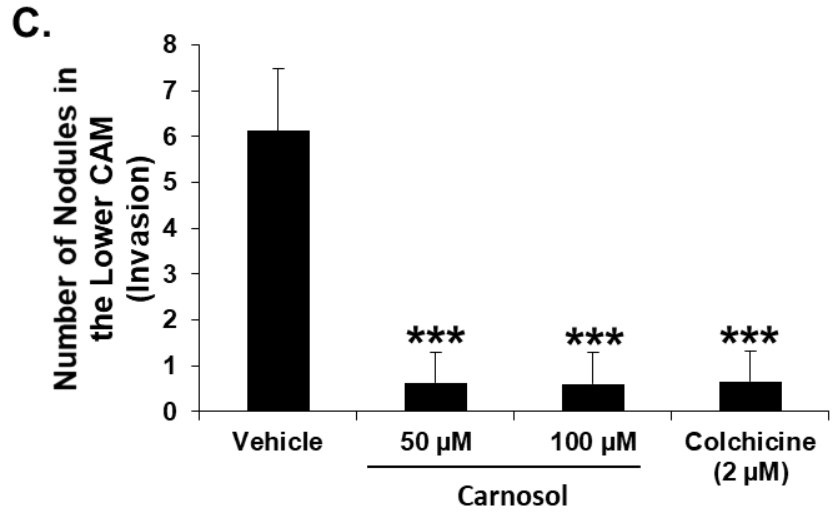


Figure 8: Antimetastatic and antitumor growth activity of carnosol on breast tumor in chick embryo chorioallantoic membrane assay (Continued).

(A) MDA-MB-231 ($1\times10^6$) cells were grafted on the CAM of 10-day chick embryo (E10). Every 48 h Tumors were treated with carnosol (50 µM and 100 µM) or 0.2% DMSO (vehicle) or colchicine (2 µM) as described in Materials and Methods. Later, tumors were collected and weighted at day 19 (E19). (B) Weight measurements of tumors in vehicle-, colchicine- and carnosol-treated chick embryo. (C) Antimetastatic effect of carnosol assessed by counting the nodules observed in the lower CAM of chick embryo treated with vehicle, colchicine or indicated concentrations of carnosol. Columns represents mean; bars represent SEM (***$p< 0.001$).

### 3.1.2 Discussion

The aim of this part of the study (section 1) was to investigate the efficacy of carnosol to inhibit tumor growth and metastasis of triple-negative breast cancer cell lines**.** The results showed that carnosol markedly reduced the growth of the tumor-derived from MDA-MB-231 cells and significantly inhibited invasion and metastasis both *in vitro* and *in vivo*. Additionally, the results have demonstrated that carnosol exerts its effect against breast cancer, at least partly, through downregulating the activity and the expression of MMP-9, inhibiting the STAT3 signaling pathway through a ROS-dependent proteasome degradation of STAT3 protein.

Cancer metastasis is a complex process involving several steps, a critical one of which is invasion, that involves proteolytic degradation of the ECM. It has been reported that elevated levels of MMPs are linked to the aggressiveness of breast cancer and metastatic potential [119,249,250]. Inhibiting the expression or activity of these proteases is considered a potential therapeutic approach against breast cancer. Here, the results in Section 1 showed that the anti-metastatic activity of carnosol involves suppressing both the expression and activity of MMP-9. This implies that carnosol is expected to reduce the degradation of the ECM.

Several signaling pathways, including STAT3, are known to regulate the expression of various genes involved in the process of tumor migration and invasion. By virtue of its ability to upregulate MMP-9, STAT3 appears to robustly enhance tumor migration and invasion of MDA-MB-231 cells. Relevantly, a study by Kim and collaborators showed that suppression of STAT3 phosphorylation by LYR71, a derivative of trimeric resveratrol, was associated with an inhibition of MMP-9 in MDA-MB-231 cells [251]. This was further confirmed by Li *et al.* [252] using pectolinarigenin and by Dees *et al.* [253] using pimozide agents to treat TNBC cell lines. Moreover, MMP-9 activation required the recruitment of STAT3 to MMP-9 promoter, and that LYR71 reduced MMP-9 transcripts by blocking STAT3 on the MMP-9 promoter [254].

Song *et al*. showed that juxtaposed STAT3/AP-1 element on the MMP-9 promoter plays a crucial role in the manner of enhancer in the activation of the MMP-9 gene. Indeed, a study reported that the functional cooperation of the STAT3 and AP-1 transcription factors is required for the transcription of the MMP-9 gene in breast cancer cells [255]. It is also noteworthy to mention that, in addition to its role in migration, invasion, and metastasis, MMP-9 is known to play a crucial role in

angiogenesis as well as tumor formation [256,257]. Most relevantly, it has been shown that the expression of MMP-9 is induced by STAT3 [267,268] . Indeed, overexpression of constitutively active form of STAT3c in breast cells led to a significant upregulation (up to 4-fold increase) of MMP-9 mRNA transcript. Added to that, these authors also showed that MMP-9 expression correlates with that of activated STAT3 in human breast cancer specimens. This is in accordance with another report that showed that the inhibition of STAT3 abolishes the activity of MMP-9 in MDA-MB-231 cells [130]. Finally, Knockdown of STAT3 in the multidrug-resistant breast cancer, SK-BR-3/EPR, cell line inhibited cell invasion and downregulated MMP-9 in these cells [258]. Interestingly, here the results showed that carnosol dramatically reduced the level of STAT3, a transcription factor required for MMP-9 expression, and downregulated MMP-9 in MDA-MB-231 cells. This led to a hypothesis stating that the depletion of STAT3 by carnosol contribute, although maybe not solely, to the inhibition of transcription of the MMP-9 gene, which results in the downregulation of MMP-9 protein expression and activity level.

Persistent activation of STAT3 has been described in several cancers, including breast cancer. A crucial role of STAT3 in tumor onset and progression, tumor cell invasion, metastasis, and angiogenesis has been largely demonstrated. Therefore, targeting STAT3 is an attractive approach given that it assaults cancer on multiple fronts [254]. Recently, carnosol was shown to attenuate activation of STAT3 signaling by inhibiting its phosphorylation while having no effect on the inactive STAT3 in HCT116 human colon cancer cells [248]. In agreement with this finding, the results of this current study (section 1) also found that carnosol inactivated STAT3 signaling pathway in a panel of breast cancer cell lines (MDA-MB-231, Hs578T, MCF-7, and T47D). Strikingly,

here we demonstrated that inactivation of STAT3 signaling in all breast cancer cell lines tested involved a different mechanism. Indeed, targeting of total STAT3 protein to proteasomal degradation was notable, hence suggesting that inhibition of STAT3 by carnosol involves a cancer-type specific mechanism(s). In recent years, several potent and selective inhibitors of STAT3 of synthetic or natural origin have been described. These inhibitors appear to act via a direct or indirect mode of action. While indirect inhibitors block the upstream effectors regulating STAT3 activation, direct inhibitors, on the other hand, block phosphorylation, dimerization, nuclear translocation, and DNA binding of STAT3 [259,260]. Several studies showed that paeoniflorin (PF), a natural compound, suppressed growth, migration, invasion, and tumor growth of U87, U251, T98G glioblastoma cells. Interestingly, PF was shown to target STAT3 to proteasome degradation [261,262]. However, the exact mechanism through which PF targets STAT3 to proteasome degradation remains unknown. Similar to PF, this current study (section 1) found that carnosol also inhibits STAT3 signaling through a mechanism involving a ROS-dependent proteasome degradation of STAT3 protein. Al Dhaheri *et al.* have previously shown that carnosol induced ROS generation in a concentration-dependent manner in MDA-MB-231 cells. Here, in this report, the results have shown that the inhibition of ROS accumulation by NAC also restored the level of STAT3 protein. This strongly suggests that carnosol-mediated the accumulation of ROS through targeting STAT3 to proteasome degradation. This contributes, at least partly, to the inhibition of cell migration, invasion, metastasis, and tumor growth of breast cancer. Further studies are needed to demonstrate the effect of carnosol on STAT3 in tumors themselves. To the best of our knowledge, carnosol is the first compound reported to specifically target STAT3 for proteasome degradation in breast cancer.

A large body of evidence supports the notion that ROS can also inhibit the metastasis of breast cancer. Baicalin, for example, a natural flavonoid, was shown to suppress migration/invasion of MDA-MB-231 cells via a ROS-mediated activation of p38/JNK signaling pathway [263]. Besides, theaflavins, natural polyphenols, were shown to inhibit migration and downregulate levels of MMP-2 and MMP-9 in MDA-MB-231 through a mechanism involving ROS generation [264]. Likewise, phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC), two phytochemicals, were shown to inhibit migration and invasion of human non-small cell lung cancer cells through a ROS-dependent mechanism [265]. Based on the findings of this study, we hypothesize that ROS might contribute, albeit not solely, to the carnosol-mediated inhibition of cellular migration, invasion, and tumor growth of TNBC through proteasomal degradation of STAT3. Thus, this work, along with Al Dhaheri's previous findings [50] further suggests that carnosol may serve as a novel and effective anticancer agent for the treatment of the highly invasive and metastatic TNBC. Finally, it is noteworthy to mention that a large number of experiments carried out on animals showed that carnosol is safe [49,56–58]. Here, this study showed that carnosol at concentrations of 50 and 100 μM has no toxicity to chick embryos.

## 3.2 Carnosol Activates UPR in a ROS-Dependent Manner in Breast Cancer Cells

### 3.2.1 Results

#### 3.2.1.1 Carnosol Induces ER-Stress Through Activating the Expression of ATF4/CHOP and Promotes ATF6α Cleavage

ROS generation has been recognized to induce ER-stress mediated cell death in variety of cancer types [266]. The phytochemical carnosol has been reported to trigger ROS production, induces activation of γH2AX, a marker of DNA damage, and causes mitochondrial membrane potential depolarization in the TNBC cell line, MDA-MB 231 [50]. This draws us to examine whether carnosol induces ER stress and activate UPR system in breast cancer.

CHOP is an ER stress marker that is known to be activated upon growth arrest and DNA damage [267]; therefore, MDA-MB-231 and Hs578T cells were treated with different concentrations of carnosol, and CHOP level was evaluated. As shown in Figure 9A (left side), CHOP protein expression increased as carnosol concentration increases. However, when cells were pretreated with NAC (a ROS scavenger) for 1 h, then treated with 50 μM and 100 μM of carnosol, the level of CHOP protein dramatically decreased (Figure 9A, right side).

CHOP, in order to be expressed and activated upon ER stress, needs the transcription factors ATF4 and cleaved form of ATF6α to be recruited to its promoter [138,139]. For this reason, we investigated the effect of carnosol treatment in ATF4 and cleaved ATF6α proteins levels in MDA-MB-231 and Hs578T cells. The results in Figures 9C and 9D revealed that at high concentrations of carnosol, 50 μM and 100 μM, ATF4

and cleaved ATF6α (N) proteins levels were increased (left side); however, both returned to a similar level as the control once treated with NAC (right side). This demonstrate that carnosol stimulated ROS production, which acts as an upstream inducer for ER stress, ultimately activates the two of the UPR sensors pathways, which are ATF4/CHOP and ATF6α.

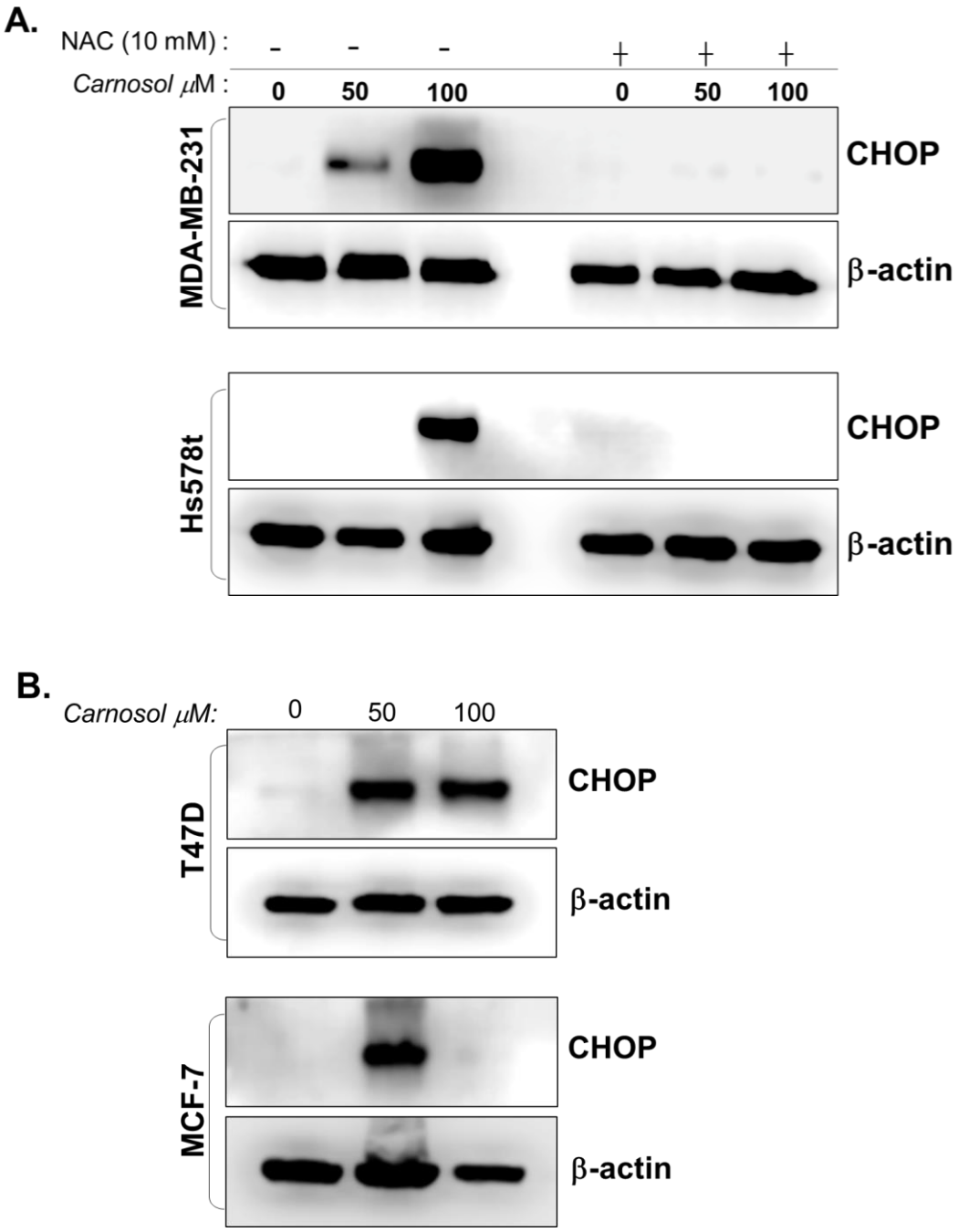


Figure 9: Carnosol induces ER-stress through activating ATF4/CHOP expression and stimulates ATF6α cleavage.

(A, C and D) MDA-MB-231 and Hs578t cells were pretreated first with a ROS scavenger, NAC, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 µM and 100 µM), and cells were incubated for 24 hours. (B) T47D and MCF-7 cells were treated with different concentrations of carnosol;50 µM and 100 µM for 24 hours. Whole cells lysate were resolved on 12.5%, 10% and 8% SDS-PAGE and examined by Western blotting for CHOP, ATF4 and cleaved ATF6α ((P) For total and (N) for cleaved ATF6α) proteins. Experiments were repeated 4 times.

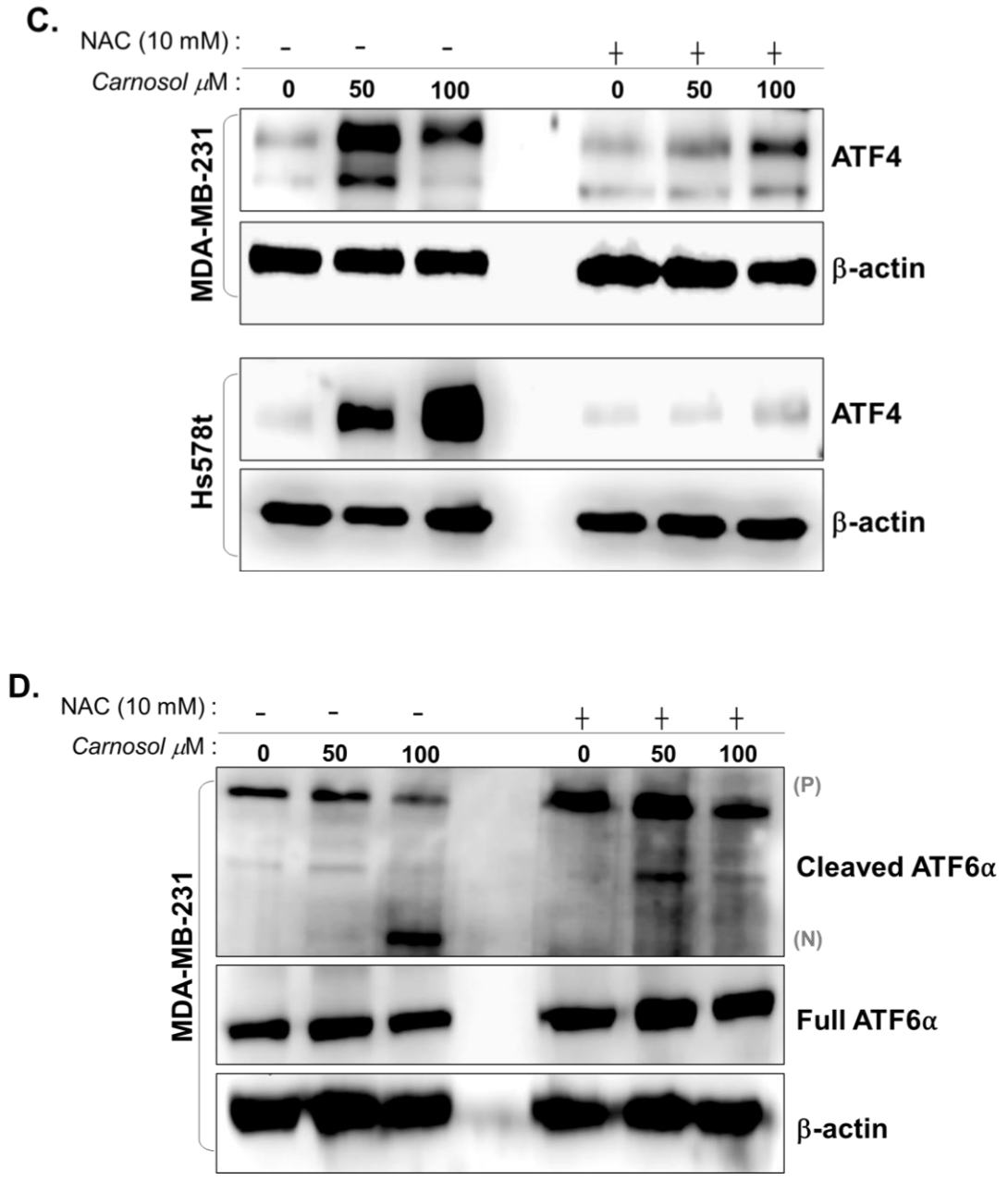


Figure 9: Carnosol induces ER-stress through activating ATF4/CHOP expression and stimulates ATF6α cleavage (Continued).

(A, C and D) MDA-MB-231 and Hs578t cells were pretreated first with a ROS scavenger, NAC, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 μM and 100 μM), and cells were incubated for 24 hours. (B) T47D and MCF-7 cells were treated with different concentrations of carnosol;50 μM and 100 μM for 24 hours. Whole cells lysate were resolved on 12.5%, 10% and 8% SDS-PAGE and examined by Western blotting for CHOP, ATF4 and cleaved ATF6α (P) For total and (N) for cleaved ATF6α) proteins. Experiments were repeated 4 times.

### 3.2.1.2 Carnosol Stimulates the Activation of IRE1α and Splicing of XBP1

As the previous results showed that carnosol stimulated the activation of UPR pathways, this drove us to investigate the effect of carnosol on the third sensor, which is IRE1α /XBP1. The phosphorylated form of IRE1α is required for splicing the mRNA of the transcription factor, XBP1, to further control the genes expression involved in protein folding and trafficking [143,144].

Therefore, an examination of the total and phosphorylated form of IRE1α in the presence of carnosol treatment in TNBC was carried out. The result in Figure 10A (left side) showed that the total and phosphorylated form of IRE1α was increased the most at 100 μM of carnosol treatment. Since phospho-IRE1α protein expression increased, this made us predict that XBP1s (the spliced mRNA) will increase as well. As expected, carnosol treatment has stimulated the splicing of XBP1, which ultimately increased the expression of spliced XBP1s (Figure 10B, left side). Moreover, to investigate whether ROS accumulation, induced by carnosol, is needed to activate the UPR sensor IRE1α / XBP1 pathway, TNBC cells were pretreated with NAC for 1 hr; then, carnosol was applied with different concentrations for 24 hr. Western Blot results (Figure 10A and B, right side) revealed that upon the NAC application, phospho-IRE1α and XBP1 protein expression levels decreased sharply to be similar to the non-treated cells. By this, carnosol has exhibited its ability to activate UPR sensors pathways, including IRE1α/ XBP1through stimulation of ROS production.

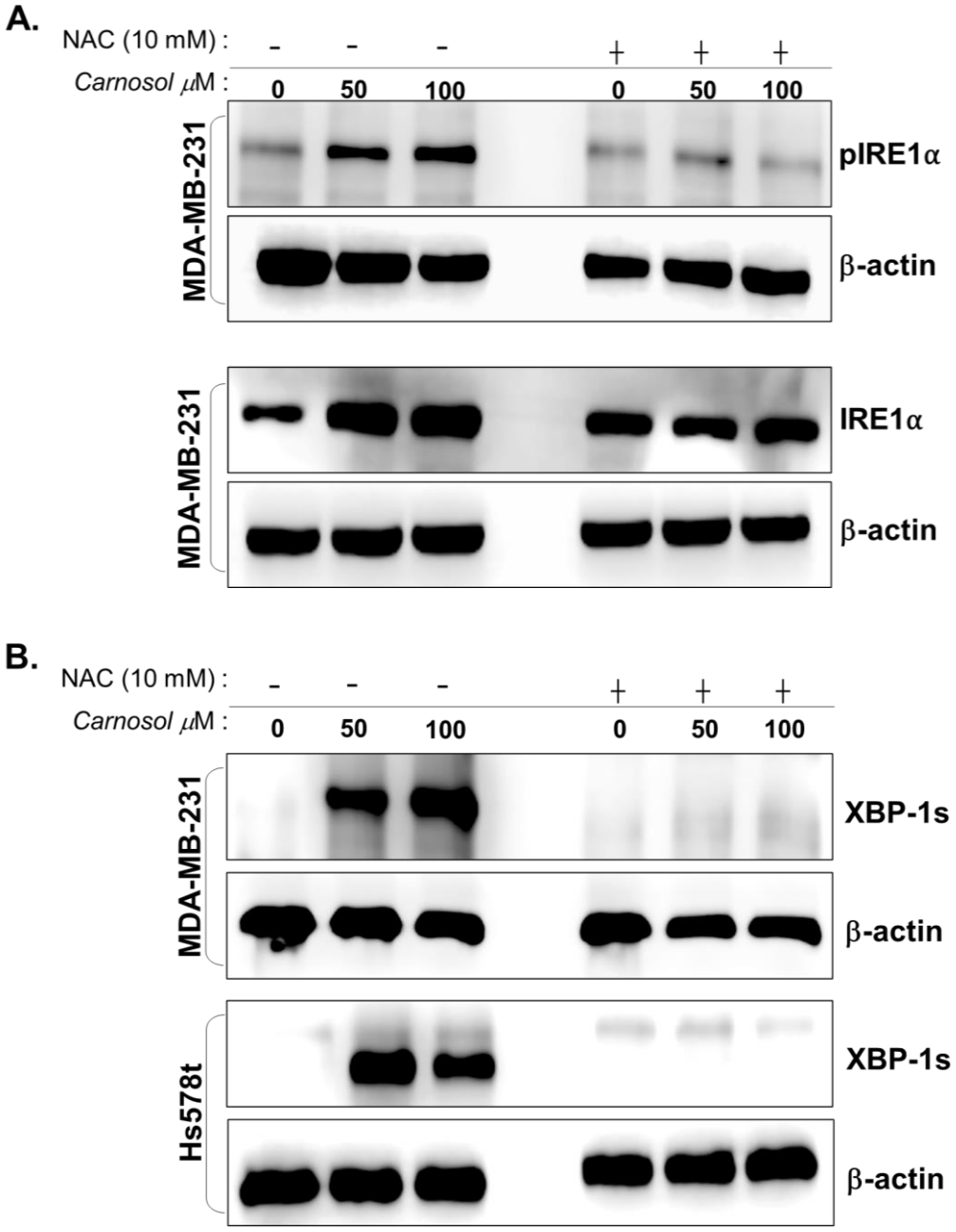


Figure 10: Carnosol stimulates the phosphorylation of IRE1α and promotes the splicing of XBP1.

(A and B) MDA-MB-231 and Hs578t cells were pretreated first with a ROS scavenger, NAC, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 μM and 100 μM), and cells were incubated for 24 hours. Whole cells lysates were resolved on 10% and 8% SDS-PAGE and examined by Western blotting for phosphorylated IRE1α, total IRE1α and XBP1s proteins. Experiments were repeated 4 times.

### 3.2.1.3 Carnosol Upregulates β-catenin and ER-Stress Chaperones

Beside ROS, β-catenin can also triggers early ER stress signaling via activating eIF2α and increasing the expression of CHOP and p21, leading to immediate growth inhibition and apoptosis in different cancers [268]. Our lab has previously shown that carnosol significantly and dramatically increases p21 protein level in TNBC cells, MDA-MB-231, causing cell cycle arrest [50]. Here in this study, we showed that CHOP level increased in MDA-MB-231 and Hs578T upon carnosol treatment Figure 9A (left side). This led to further evaluate the level of β-catenin in TNBC cells treated with the carnosol. We found that Carnosol treatment caused a dramatic rise in the expression of β-catenin in MDA-MB-231 cells (Figure 11A, left side); however, when cells pretreated with NAC prior to addition of carnosol, β-catenin protein level sharply declined (Figure 11A, right side). This result strongly suggest that ROS generation is an early event that induce β-catenin expression, consequently, triggers early ER stress.

ER lumen chaperons and specialized enzymes are important to ensure protein folding and trafficking. When misfolded proteins get accumulated upon stress, ER chaperon proteins such as BiP will be released form the UPR sensors, holding the misfolded proteins and letting the UPR to be activated. Hence, the level of BiP protein was examined in cells treated with increasing concentration of carnosol. The result in Figure 11B (left side) showed that the molecular chaperone Bip was increased by carnosol in a dose-dependent manner. On the other hand, when ROS accumulation was blocked by NAC, BiP expression in carnosol-treated cells was comparable to non-treated cells as shown in Figure 11B (right side side).

Calnexin, Ero1-Lα and Protein disulfide isomerase (PDI) are ER specialized enzymes that function in glycoprotein quality control, redox generation, and protein disulfide isomerase, respectively. Therefore, we decided to study the effect of carnosol on these specialized enzymes. Carnosol induced the unfolding protein response by downregulating the protein expressions of PDI and Ero1-Lα (Figure 11C & 11D, left side). However, no significant change was observed in the level of the calnexin as in Figure 11E. Overall, this data indicates the impact of carnosol and its involvement in regulating ER chaperons' expression.

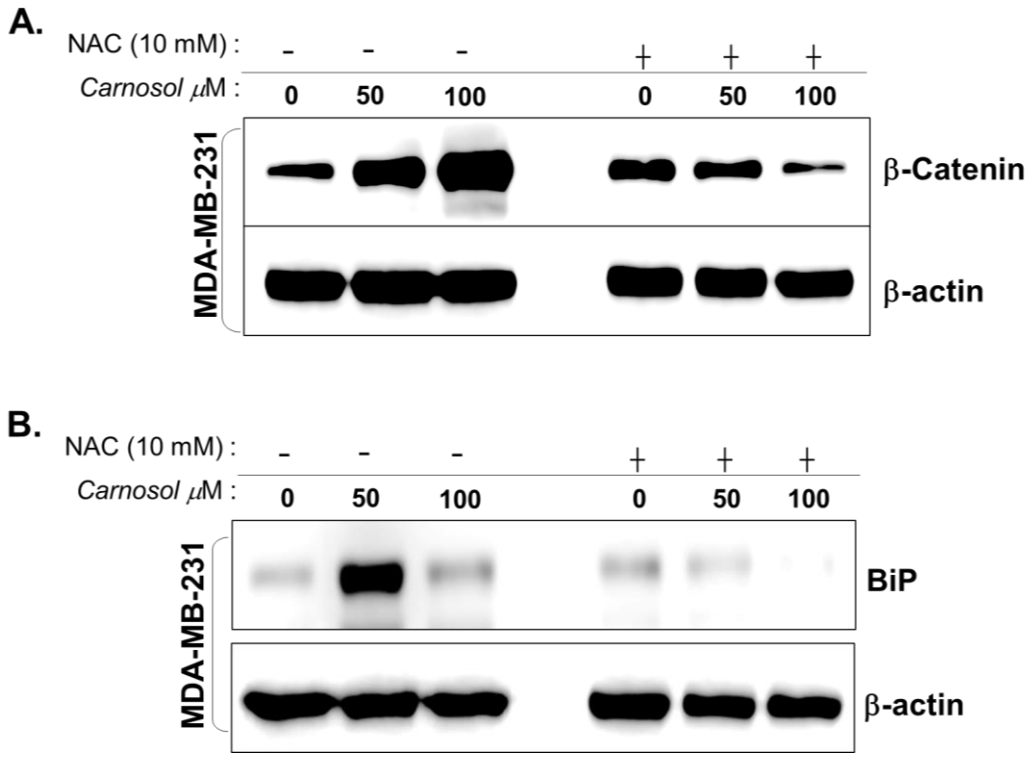


Figure 11: Carnosol upregulates β-catenin and ER-stress chaperones.

(A, B, C, and E) MDA-MB-231 cells were pretreated first with a ROS scavenger, NAC, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 μM and 100 μM), and cells were incubated for 24 hours. Whole cells lysates were resolved on 10% and 8% SDS-PAGE and examined by Western blotting for β-catenin, BiP, PDI, Ero1-Lα and calnexin proteins. Experiments were repeated 4 times.

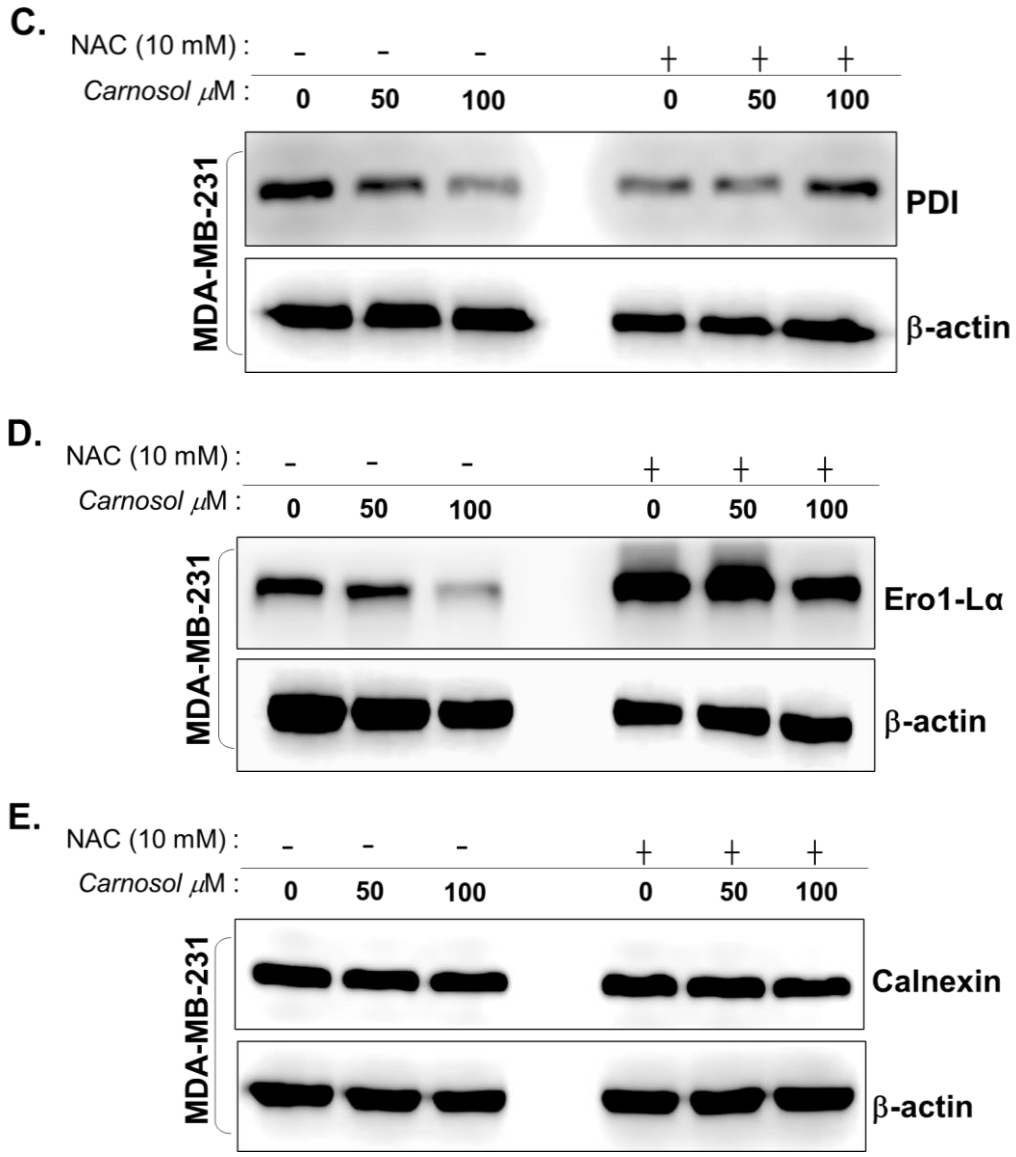


Figure 11: Carnosol upregulates β-catenin and ER-stress chaperones(Continued).

(A, B, C, and E) MDA-MB-231 cells were pretreated first with a ROS scavenger, NAC, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 μM and 100 μM), and cells were incubated for 24 hours. Whole cells lysates were resolved on 10% and 8% SDS-PAGE and examined by Western blotting for β-catenin, BiP, PDI, Ero1-Lα and calnexin proteins. Experiments were repeated 4 times.

#### 3.2.1.4 Carnosol-Induced Oxidative Stress Inhibits the Cell Survival Signaling Akt/mTOR and Activates Protein Degradation Through Ubiquitination

Carnosol has shown to inhibit cell survival, induce apoptosis and autophagy in MDA-MB-231 cells [50]. Therefore, we sought to examine the expression of Akt /mTOR signaling, a major pathway in the growth and survival of cancer cells and inhibitor of autophagy. Carnosol observes to downregulates Phospho-Akt and total-Akt expression in MDA-MB-231 cell line at 24 h by immunoblotting (Figure 12A, left side). Pretreatment of the cells with NAC for 1 h, and then cotreating them with carnosol for 24 h resulted in a significant increase in phospho-Akt and total-Akt in MDA-MB-231 cells as shown in Figure 12A, right side). Analysis of phospho-mTOR and total-mTOR level in carnosol treated cells by Western Blotting was consistent with Akt results (Figure 12B). There was a highly significant reduction in phospho-mTOR and total-mTOR in breast cancer cells when treated with carnosol (Figure 12B, left side), but blocking ROS with NAC during carnosol treatment restores phospho-mTOR expression (Figure 12B, right side). These outcomes suggest the anticancer effect of carnosol on the cell survival signaling pathways.

Zhoa *et al*, showed that a decrease in mTOR activity increases overall protein ubiquitination and degradation by the ubiquitin proteasome system [269] . This provoked the author to examine whether inactivation of mTOR activity by carnosol enhances overall protein ubiquitination. MDA-MB-231 cells were treated with 50 and 100 μM carnosol and overall protein ubiquitination level was determined by Western Blotting using ubiquitin antibody. The result revealed that treatment with carnosol caused a marked increase in the total content of ubiquitinated proteins (Figure 12C). However, when MDA-MB-231 cells were pretreated first with NAC for 1 h then carnosol was

added, the overall protein ubiquitination level dramatically decreased. This result implies a prosurvival pathway by dragging proteins to be degraded by proteasome through ubiquitination.

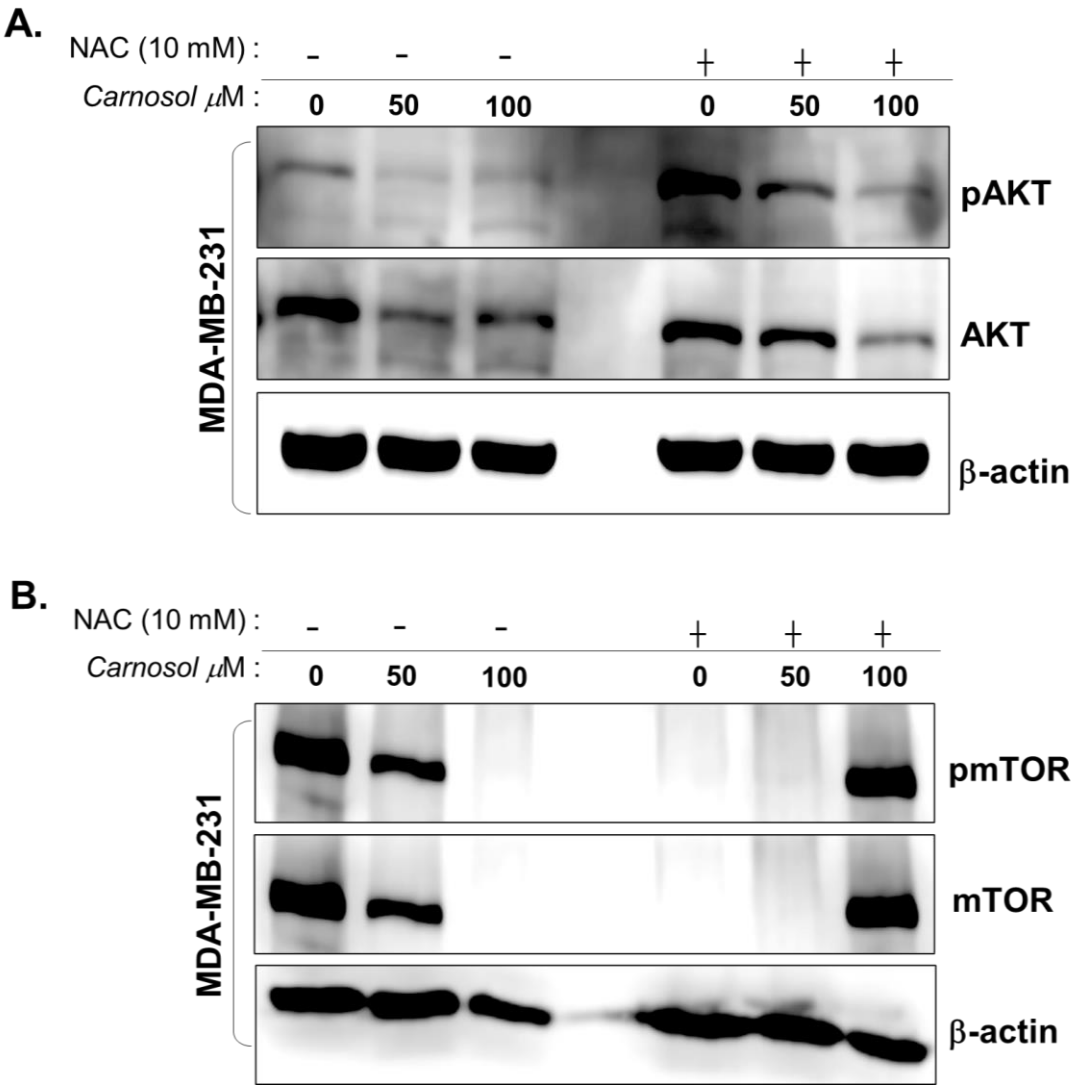


Figure 12: Carnosol-induced oxidative stress constrains the cell survival signaling Akt/mTOR and activates protein degradation through ubiquitination.

(A, B, and C) MDA-MB-231 cells were pretreated first with a ROS scavenger, NAC, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 µM and 100 µM), and cells were incubated for 24 hours. Whole cells lysates were resolved on 12.5%, 10% and 8% SDS-PAGE and examined by Western blotting for phosphorylated AKT, total AKT, phosphorylated mTOR, total mTOR and ubiquitinated proteins. Experiments were repeated 3 times.

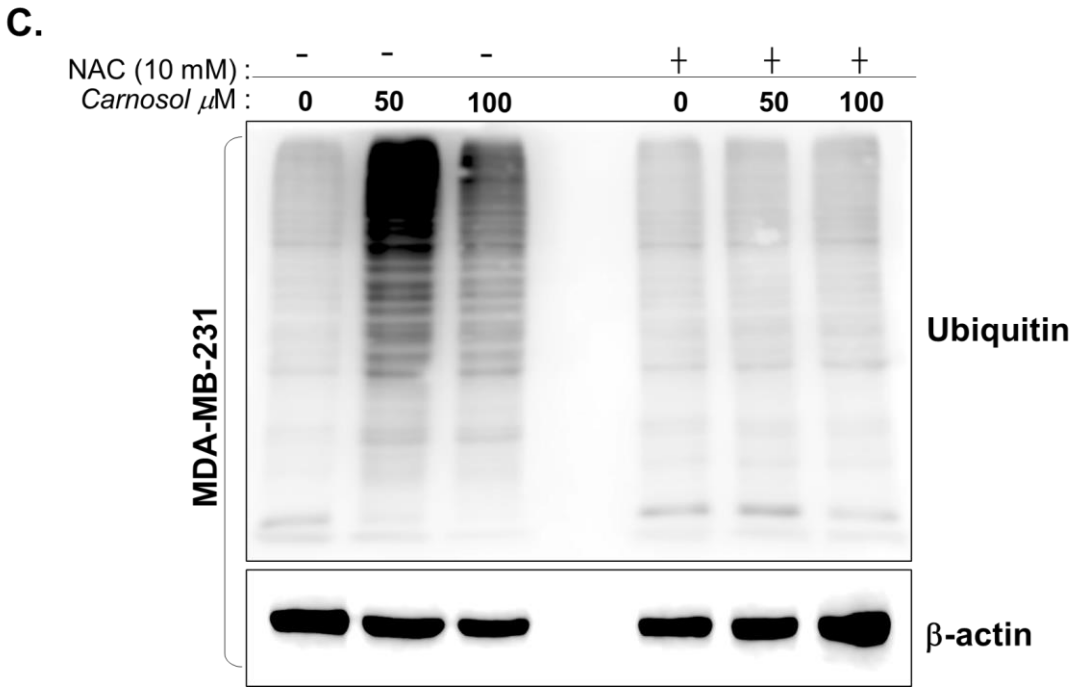


Figure 12: Carnosol-induced oxidative stress constrains the cell survival signaling Akt/mTOR and activates protein degradation through ubiquitination (Continued).

(A, B, and C) MDA-MB-231 cells were pretreated first with a ROS scavenger, NAC, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 µM and 100 µM), and cells were incubated for 24 hours. Whole cells lysates were resolved on 12.5%, 10% and 8% SDS-PAGE and examined by Western blotting for phosphorylated AKT, total AKT, phosphorylated mTOR, total mTOR and ubiquitinated proteins. Experiments were repeated 3 timeS.

### 3.2.1.5 Carnosol Regulate Cell Death via Upregulating the Expression of P38

One of the most significant target of cancer treatment is inducing cell death, and several studies have demonstrated the involvement of the MAPK P38 in regulating the balance or switch between autophagy and apoptosis in cancer upon the induction of ER stress [166],[270,271]. Our lab previously showed that carnosol induced autophagy followed by activation of apoptosis in MDA-MB-231 cells. We sought to examine the p38 MAPK pathway in carnosol-treated cells. Figure 13A (left side) shows a dose-dependent increase in phosphor-p38 in carnosol-treated MDA-MB-231 cells.

However, the ROS scavenger, NAC completely abolished p38 activation (Figure 13A, right side). Other breast cancer cell lines, T47D and MCF7 (Figure 13B), showed an increase in the pP38 level as the MDA-MB-231 cells. This result suggests a possible involvement of P38 activation in carnosol-mediated ER stress, autophagy and/or apoptosis. To further understand the mechanism of p38 involvement in cell death decision, breast cancer cells were pretreated with p38 inhibitors (SB 203580 and SB 202190), for 1 h and carnosol was applied and then cleaved PARP (marker of apoptosis) and LC3 II (marker of autophagy) protein level was assessed. As shown in Figure 13C LC3 II level decreased in cell treated with p38 inhibitors in comparison to control, suggests that p38 activation plays an important role in carnosol-induced autophagy. Conversely, inhibition of p38 had no effect on the activation of apoptosis. Apoptosis, demonstrated by cleaved PARP, occurred in cell treated with p38 inhibitors at level comparable to control cells (Figure 13D). Altogether, our results suggest that p38 activation is involved in the process of activation of autophagy and not apoptosis. A putative downstream target of p38 is CHOP, which mediates ER stress-induced autophagy, apoptosis, or inflammation [272]. Since CHOP was found previously to be stimulated with carnosol treatment, this prompted us to evaluate CHOP level in cells pretreated with SB 203580 and SB 202190 and then cotreated with carnosol for 24 hr. Inhibiting p38 activation has sharply reduced the protein level of CHOP (Figure 13E). Hence, we hypothesized that p38 might play a critical role in carnosol-induced CHOP upregulation which in turn induces autophagy.

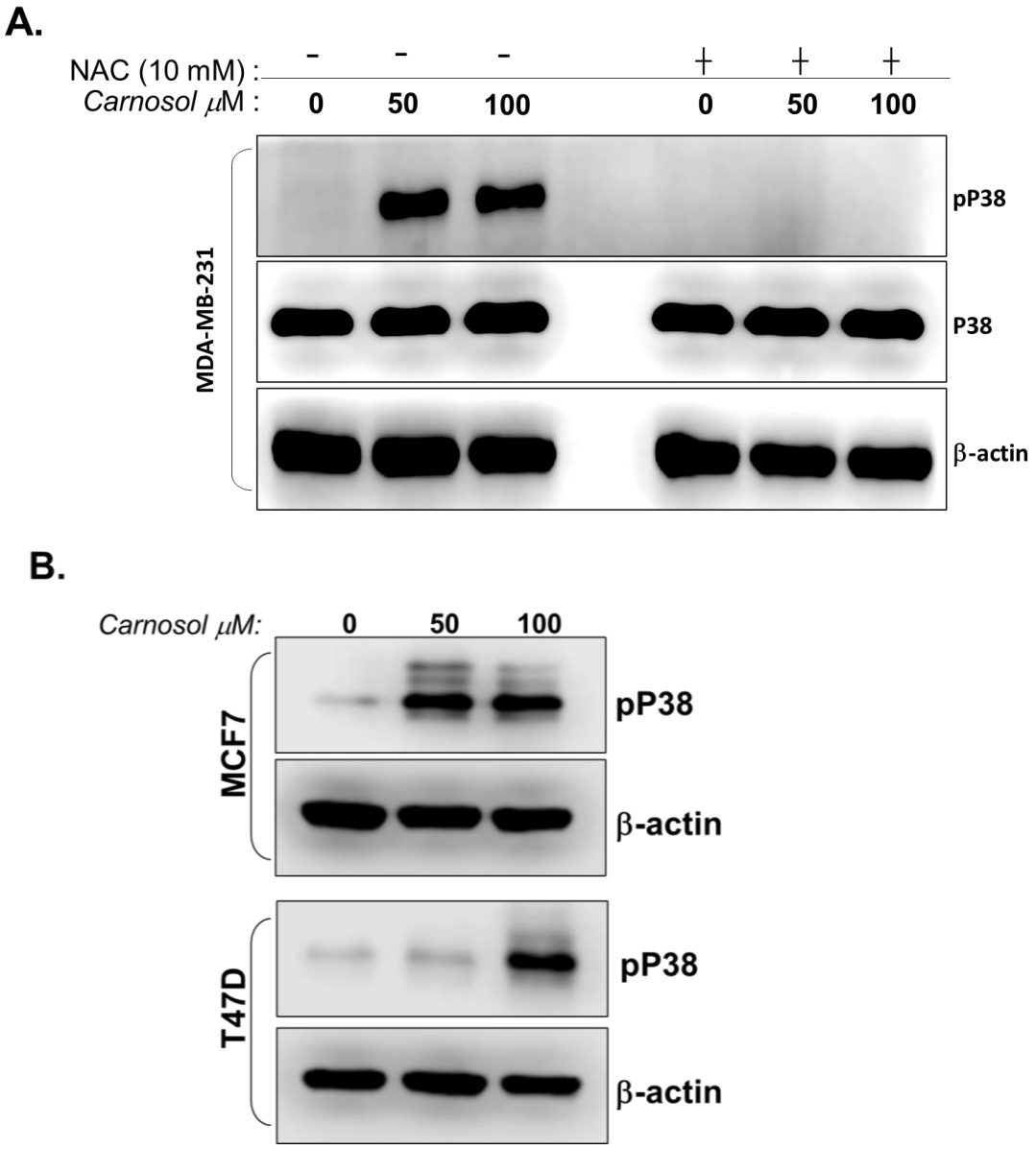


Figure 13: Carnosol control cell death through upregulating P38 expression.

(A) MDA-MB-231 cells were pretreated first with a ROS scavenger, NAC, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 µM and 100 µM), and cells were incubated for 24 hours. (B) MCF7 and T47D cell were treated with 50 µM and 100 µM of carnosol and incubated for 24 hours. (C, D and E) MDA-MB-231 cells were pretreated first with P38 inhibitors, SB 203580 and SB 202190, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 µM and 100 µM), and cells were incubated for 24 hours. Whole cells lysates were resolved on 12.5% and 10% SDS-PAGE and examined by Western blotting for phosphorylated P38, total P38, LC4I/II, cleaved PARP and CHOP proteins. Experiments were repeated 3 times.

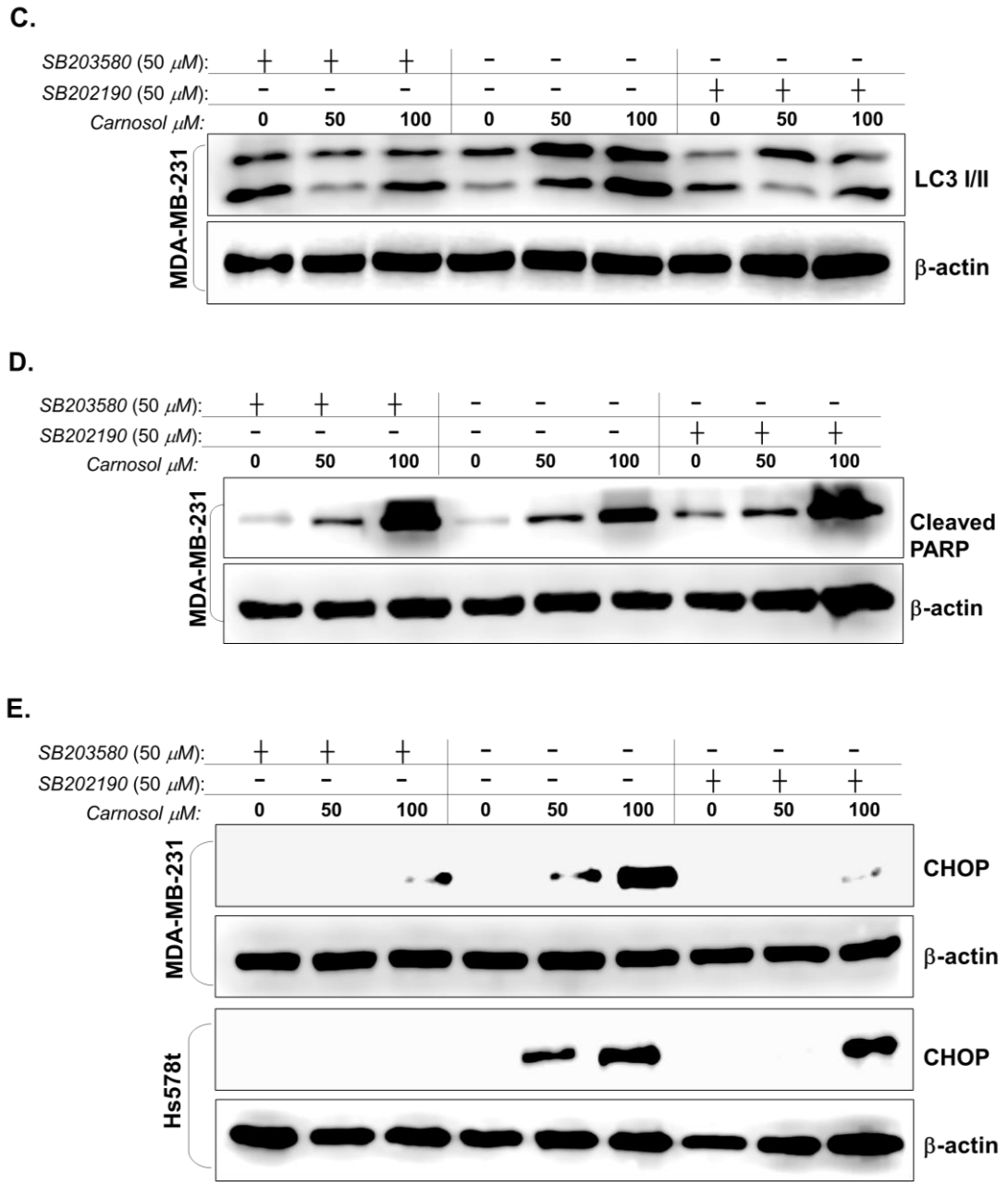


Figure 13: Carnosol control cell death through upregulating P38 expression (Continued).

(A) MDA-MB-231 cells were pretreated first with a ROS scavenger, NAC, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 µM and 100 µM), and cells were incubated for 24 hours. (B) MCF7 and T47D cell were treated with 50 µM and 100 µM of carnosol and incubated for 24 hours. (C, D and E) MDA-MB-231 cells were pretreated first with P38 inhibitors, SB 203580 and SB 202190, and vehicle (0.2% DMAS) for 1 hour, then, different concentrations of carnosol were added (50 µM and 100 µM), and cells were incubated for 24 hours. Whole cells lysates were resolved on 12.5% and 10% SDS-PAGE and examined by Western blotting for phosphorylated P38, total P38, LC4I/II, cleaved PARP and CHOP proteins. Experiments were repeated 3 times.

### 3.2.2 Discussion

Studies have showed that some cancers are more sensitive to ROS than normal cells [149], thus, targeting ROS induction could be an effective approach for killing cancer cells selectively without causing significant toxicity to normal cells [154,155]. Additionally, several reports have discussed the beneficial effects of ROS production on chemotherapy-induced cell death in cancer cells [147–149]. Therefore, finding a new drug that stimulates ROS production and ultimately leads to UPR activation is considered a goal for new effective therapeutic approaches in cancer treatment.

Currently used anticancer therapeutics have represented limitations due to unfavorable side effects and drug resistance [273]. Thus, scientists need to find new agents to develop and expand more reliable treatments. Plant-derived drugs are showing curative anticancer potential, hence, clarifying how these natural compounds interact with cellular targets is important. Affecting the increase of ER stress and UPR related proteins may perhaps represent an interesting approach to modify the homeostasis of the ER in cancer cells to activate autophagy and apoptosis. Numerous natural compounds have been shown to induce ER stress-related cells death in malignant cells [274]. For example, saxifragifolin D (found in *Androsace umbellate*) was reported to induces ROS which in turn leads to growth inhibition via apoptosis [275]. Another study has showed that a curcuminoid derivative called MTH-3 induced apoptosis intrinsically and extrinsically by ER stress signals in MDA-MB-231 human breast cancer cell line [276]. Patacsil *et al.* revealed that γ-tocotrienol (a vitamin E compound) has stimulated ER stress-mediated apoptosis as verified by gene expression microarray analysis in breast cancer cell lines, MDA-MB-231 and MCF-7. Indeed, microarray results highlighted the modulation of genes expression involved in the ER stress

response, such as ATF3 (a target gene for ATF4); the authors showed that ATF3 had a critical role in γ-tocotrienol-induced apoptosis in MCF-7 cell line. Additionally, γ-tocotrienol has upregulted the ER stress-related protein markers BiP, PERK , ATF4 and IRE1α in both MDA-MB-231 and MCF-7 cells [277]. A study by Aghaei *et al*. has demonstrated the antibreast cancer activity of pimpinelol (a lactone found in *Pimpinella haussknechtii*), where it induces apoptosis via upregulating the protein expression involve in ER stress and increase protein aggregation. Results were shown using fluorescence microscopy analysis and mRNA expression of ATF4, CHOP, GADD34, and TRIB3 [278]. These studies proposed a promising potential of how natural compounds can act as important chemical molecules that are able to modulates and induces ER stress to further cause cancer cell death.

In this part of the study (2nd section), the polyphenolic compound, carnosol, was shown to induce ROS-dependent ER stress through upregulating the three main UPR sensor pathways and their downstream targets which are ATF4/CHOP, ATF6α and IRE1α/XBP1. Moreover, β-catenin found to be stimulated by carnosol, and considered to be an upstream trigger of ER stress. In addition, a reduction in the cell survival signaling Akt/mTOR was observed with carnosol treatment which consequently activates protein degradation through ubiquitination.

The UPR is a cellular stress response that start in the ER, and controlled by three different sensors, IRE1α, PERK, and ATF6α. Usually the ER resident chaperones, such as BiP is bound to the ER luminal domain of the three sensors, retaining them in an inactive state [279]. Under stress conditions that lead to accumulatedunfolded or misfolded proteins causing an ER stress, BiP detach from IRE1α and PERK, letting the sensors to form homodimers or oligomers, and then through autophosphorylation,

it activates their downstream signals. Also, the release of BiP cause the cleavage and activation of ATF6α to transfer to the nucleus and activates the transcription of different ER chaperones [280].

In response to UPR activation, PERK gets activated, consequently attenuates the translation of mRNA and preventing the delivery of new proteins into the ER compartment. This action is mediated by the activation of eIF2α which block proteins translation [281], but not absolute blockade. Indeed, in the meantime, active eIF2α paradoxically positively regulates the translation of the transcription factor ATF4 [139]. In turn, ATF4 regulates the expression of genes implicated in the survival and restoration of normal cellular homeostasis such as CHOP [282]. The CHOP gene, in contrast to ATF4, it upregulates the expression of genes involve in growth arrest, DNA damage inducible genes and apoptotic pathway [283,284]. ATF4 considered as a key signal for autophagy-induced by ER stress, and subsequent upregulation of CHOP switches autophagy into apoptosis. This switch is happening between ATF4 and CHOP in the PERK cascade [285,286]. Upregulation of ATF4 and CHOP were found to induces autophagy in different cancer types [287,288]. Another study showed that the knockout of CHOP protein in mice model have revealed a lower apoptotic rate for ER stress response [289]. Here, carnosol has been found to induce the upregulation of ATF4/CHOP in breast cancer cells in response to ROS induction. This action was further evaluated by treating the cells with NAC, a ROS scavenger, were the expression of ATF4/CHOP was completely abolished.

IRE1α is another pathway which gets activated upon ER stress, BiP dissociate from IRE1α receptor letting dimerization and autophosphorylation to happen, and consequently converting IRE1α into its active state. Active IRE1α triggers its

endonuclease activity to splice the mRNA of XBP1, which encodes for a transcription factor targeting genes involved in prosurvival responses [290], enhances protein folding, transport and functions, and resolves misfolded proteins [291,292]. On the other hand, active IRE1α triggers another downstream signal through post-transcriptional modification of different substrates that tends to trigger apoptosis [293]. When ER stress occurs, IRE1α gets activated rapidly, and when ER stress is transformed to chronic stress, the signal of IRE1α is weakened [294,295] through dephosphorylation, ubiquitination and degradation [296]. Breast cancer cells have been treated with carnosol and the activation of IRE1α has been evaluated by Western Blotting. IRE1α phosphorylation showed an increase in a concentration dependent manner with carnosol treatment, meanwhile, an addition of NAC eliminates the activation, suggesting the importance of ROS in the induction of UPR.

ATF6 has two homologous which are ATF6α and ATF6β. Following UPR activation, ATF6α moves to the Golgi apparatus where it is cleaved by the proteases site-1 (S1P) and site-2 (S2P) and activated. Active (cleaved) ATF6α translocates to the nucleus and upregulates the transcription of XBP1 mRNA and then collaborates with IRE1α to produce a spliced, mature XBP1s mRNA [297]. XBP1 binds to ATF6 forming a complex that is involved in the expression of ER chaperones [298] to further stimulate protein folding, secretion and ER-related protein degradation in parallel pathways [299,300(p. 1)]. While ATF6 activity is mainly to act as prosurvival signal, during severe and prolonged ER stress, it can enhance CHOP expression that is associated with cell death [301]. In agreement with these reports, we also found that carnosol induced an upregulation of CHOP.

The proper protein folding, and assembly is supported and monitored by a number of ER resident proteins including chaperones, glycosylating enzymes, and oxidoreductases. One of the fundamental ER chaperones is BiP (Grp78) which responsible for the activation of the three main sensors and bind to misfolded proteins to engage an optimal UPR response [302]. BiP has been required for cancer cell survival, proliferation and angiogenesis [303], besides, several studies have reported an upregulation of BiP in solid tumors in various organs [304–306]. PDI is another chaperon that function as a thioldisulfide oxidoreductase and catalyze disulfide bonds in newly synthesized proteins [307]. Findings on the role of PDI in cancer have illustrated a prooncogenic and prosurvival function in addition to resistance to cancer therapy [303]. Lovata and collaborators revealed that treating melanoma cells with bacitracin, a PDI inhibitor, enhanced the stress response and the induction of apoptosis in the presence of chemotherapeutic drugs [308]. An additional ER resident protein is Ero1-Lα, a protein disulfide oxidase that has a role in the formation of disulfide bonds of cell-surface and secreted proteins. It has been reported that Ero1-Lα is highly upregulated in different types of cancers and found to be associated with breast cancer poor prognosis [309]. Targeting the previously discussed ER chaperons and resident proteins is a promising approach to treat cancer. Thus, the expression of BiP, PDI, Ero1-Lα and calnexin have been assessed upon carnosol treatment, BiP was increased while PDI, Ero1-Lα were decreased, while calnexin was constantly expressed, suggesting their involvement in UPR and inducing cell death in breast cancer.

β-Catenin is an essential component of the WNT signaling pathway [310], and has been implicated in cell proliferation and migration of multiple myloma and was investigated as a promising therapeutic target [311,312]. A study by Raab *et al.* revealed that β-catenin

induces apoptosis through activating UPR. An inhibition of protein kinase C (PKC) leads to accumulation of β-catenin by inhibiting phosphorylation which is necessary for its proteasomal degradation. Consequently, accumulated β-catenin leads to growth arrest via the induction of UPR which ultimately leads to apoptosis [268]. Interestingly, we found that carnosol dramatically upregulated β-catenin through a ROS-dependent mechanism. Hence, it is legitimate to speculate that β-catenin might be involved in carnosol-induced ER stress, autophagy and/or cell death.

The Akt/mammalian target of rapamycin (mTOR) cascade is one of the most frequently activated signaling pathways in human cancer [313].The activation of Akt is through phosphorylation, phospho-Akt inhibits TSC1/2 leading to activate mTOR. mTOR is kwon to regulate overall protein turnover and thus promoting cellular growth and proliferation. Moreover, the Akt/mTOR cascade is frequently hyperactivated in many cancers, including breast cancer [314],' and is important for aggressive tumor growth and cell survival. A study by Woo and collaborators, investigated the inhibition of Akt/mTOR pathway in breast cancer cells using a combination therapy (MK-2206, Akt inhibitor and rapamycin, mTOR inhibitor). The results have dementated the induction of apoptosis *in vitro* and inhibited tumor growth *in vivo,* suggesting that Akt/mTOR has emerged as potential target for breast cancer therapy [315]. During the activation of UPR, autophagy has been shown to be an adaptive response, in order to help cells to restore ER homeostasis, or promoting to ER stress-induced cell death [289,316]. A study by Zhaou and coauthors have indicated that ampelopsin (a flavonol mainly found in Ampelopsis grossedentata) induced ER stress, which consequently leading to protective autophagy by downregulating Akt/mTOR pathway. This suggest that targeting the cascade of ER stress activation through targeting Akt/mTOR may be

a useful strategy in treating breast cancer [317]. Interestingly, we found that carnosol downregulated the level of total mTOR and total AKT probably through targeting these two proteins to proteasome degradation. Indeed, we showed that carnosol induces an increase in the overall level of ubiquitinated of proteins suggesting an activation of the ubiquitin proteasome system.

One of the most important targets of cancer therapy is inducing cell death, and p38 is known to be involved in regulating the balance or switch between autophagy and apoptosis in cancer upon the induction of ER stress [166],[270,271]. Liu *et al.* studied the anticancer effects of Shikonin, a botanical drug extracted from *Lithospermum erythrorhizon*, in human melanoma A375 cells. They found that Shikonin induced p38 activation via ROS-mediated ER stress, leading to protective autophagy at the beginning; later, accumulated damage in the cells induced apoptosis [318]. In addition, Jiang *et al.* revealed that selenite induced a switch to apoptosis from autophagy via p38 pathway in human leukemia NB4 cells. This happens when selenite induces the disassociated of p38 from the PERK complex, and p38 becomes phosphorylated, which consequently leads to the activation of eIF2α. Hence, this event enhanced the binding of ATF4 to the CHOP promoter, which ultimately led to apoptosis. On the other hand, p38 hindered the activation of eIF4E, which inhibited the association of ATF4 with the MAP1LC3B promoter, resulting in autophagy suppression [319]. This study showed how p38 has a differential modulation mode of activation for eIF2α and eIF4E, affecting ATF4 binding to the promoters of target genes. Therefore, p38 has influenced the transcription of essential proteins and the switch from autophagy to apoptosis.

To date, several pieces of evidence have shown that some of the polyphenols [274,318,320] or natural compounds [319] exhibit their anticancer activity through ROS-induced ER stress and involvement of p38 activation in regulating cell death. Additional, preclinical studies reveal that bioactive dietary polyphenols exert anticancer effects by inducing ROS-mediated cytotoxicity in cancer cells [321]. This attracted the interest to further investigate the polyphenolic compound carnosol, as it has been proved that it induces ROS-mediated autophagy and apoptosis in TNBC [50]. Nevertheless, the role of carnosol in ROS-activate p38 is still vague and finding the correlation will guide the search for new targeted drugs for the aggressive TNBC. These studies directed the research toward investigation of the P38 activity in carnosol induce cell death. The data in this current study showed that carnosol has downregulated phospo-P38 via a ROS-dependent mechanism. Then, the effect of inhibiting P38 activity was carried out by using P38 inhibitors (SB 203580 and SB 202190), and the result reveled that p38 activation plays an important role in carnosol-induced autophagy. On the other hand, loss of p38 resulted in apoptosis enhancement by increasing cleaved PARP protein. This proposed that breast cancer cells treated with carnosol, involve P38 more in inducing autophagy than apoptosis. Strikingly, inhibition of p38 activation by the two inhibitors blocked carnosol-induced ER stress as no CHOP activation was seen in SB 203580 and SB 202190-treated MDA-MB-231 cells.

Overall, carnosol was shown to inhibit breast cancer cells through induction of ER-stress followed by autophagy and subsequent activation of apoptosis.

## 3.3 Carnosol is a Novel Inhibitor of p300 Acetyltransferase in Breast Cancer

### 3.3.1 Results

#### 3.3.1.1 Carnosol Induced Histone Hypoacetylation in Breast Cancer Cells

HATs are becoming an important target for cancer treatments as it is involved in cancer progression pathways [322], hence this derives the authors for further studying the anti-cancer effect of carnosol in triple-negative breast cancer cell lines. The overall acetylation status of histone H3 and histone H4 in carnosol-treated breast cancer cells was examined. The results revealed that carnosol induced an overall decreased in the acetylation level of histone H3 and H4 in MDA-MB-231 (Figure 14A) triple-negative breast cancer cells. Thereafter, an assessment of the acetylation status of specific lysine residues known to be substrates for acetylation was carried out using antibodies against acetylated H3K56, H3K14, H3K9, H4K16, and H4K5. Carnosol showed a differential effect on the acetylation of different residues (Figure 14B). While carnosol had little impact on the acetylation of H4K5 and H4K16, it dramatically reduced the level of acetylation of H3K56, H3K9, and H3K14 in MDA-MB-231 cells (Figure 14B). To rule out that the carnosol-induced histone hypoacetylation is a result of upregulated HDAC activity, MDA-MB-231 cells were first pre-treated with the HDAC inhibitor, Trichostatin A (TSA), and then treated with carnosol for 24 h. As shown in Figure 14C, inhibition of HDAC activities by TSA had no effect on carnosol-induced histone hypoacetylation of histone H3K56. Therefore, these results suggest that carnosol might induce histone hypoacetylation by affecting the activity of histone acetyltransferase(s) in breast cancer cells. Additionally, considering that carnosol induced histone hypoacetylation at specific residues suggests that it exerts its effect on particular HATs.

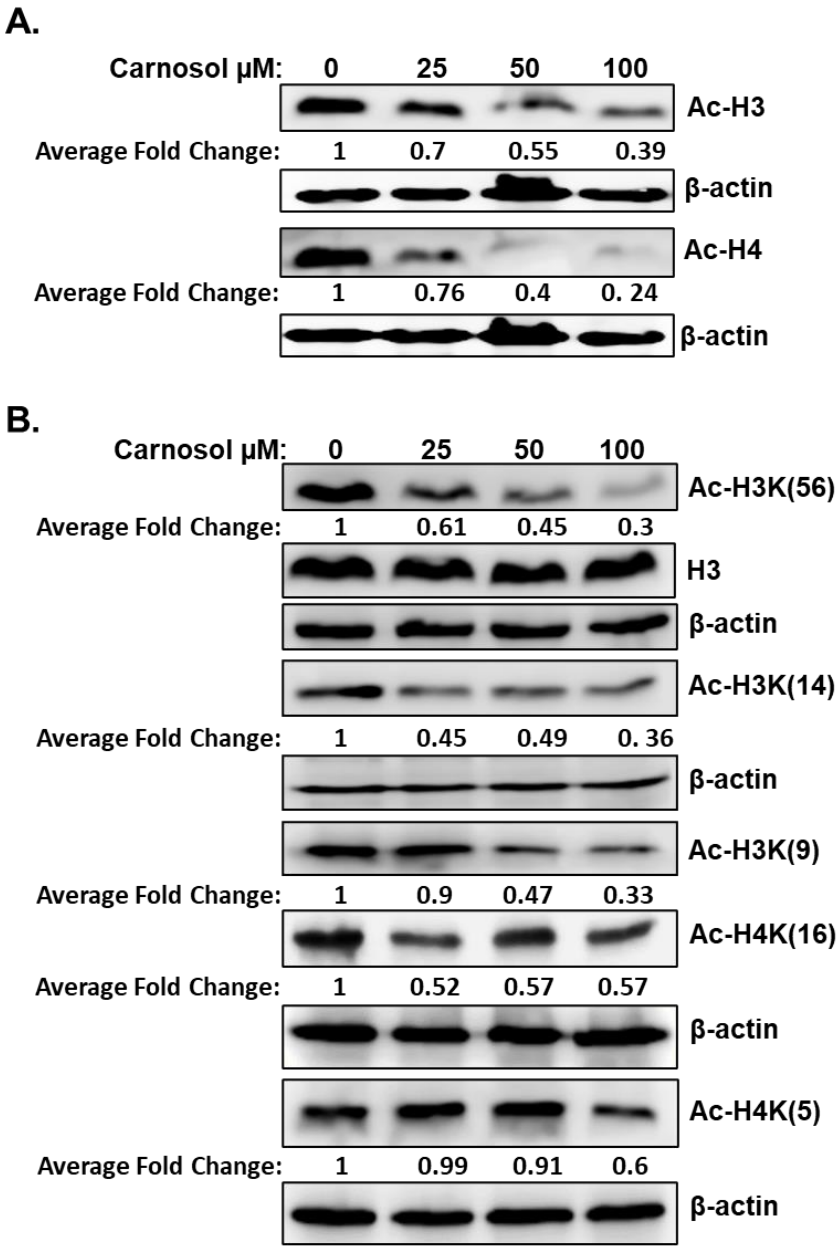


Figure 14: Carnosol stimulates histone hypoacetylation in vivo in breast cancer cells.

(A) Carnosol promotes overall histone H3 and H4 hypoacetylation. MDA-MB 231 cells were treated with vehicle (0.2% DMSO) or with carnosol (50 µM and 100 µM), then cells were collected, and proteins were extracted and subjected to Western Bloting analysis for the acetylated histone H3 and histone H4. (B) Carnosol induces histone hypoacetylation at specific residues. All cells were collocated, and proteins were extracted and subjected to Western Blotting analysis for the acetylated H3K56, H3K14, H3K9, H4K16 and H4K5. (C) HAT, and not HDAC, are implicated in carnosol-induced histone hypoacetylation. Trichostatin A (TSA) has no influence on carnosol-induced histone H3K(56) hypoacetylation, one of p300 substrates. Cells were pre-treated with or without TSA (400 nM) for 4 hours and then treated with carnosol (50 and 100 µM) for 24 hours. Whole cell lysate was resolved on 15% SDS gel and probed with acetyl-H3K56 antibody. Data shown are representative of three independent experiments. carried at different times using different protein preparations. Bands intensities were quantified using the ImageJ software (National Institute of Health, USA).

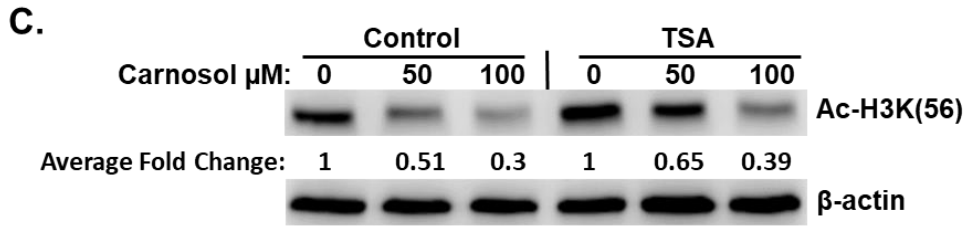


Figure 14: Carnosol stimulates histone hypoacetylation in vivo in breast cancer cells (Continued).

(A) Carnosol promotes overall histone H3 and H4 hypoacetylation. MDA-MB 231 cells were treated with vehicle (0.2% DMSO) or with carnosol (50 µM and 100 µM), then cells were collected, and proteins were extracted and subjected to Western Bloting analysis for the acetylated histone H3 and histone H4. (B) Carnosol induces histone hypoacetylation at specific residues. All cells were collocated, and proteins were extracted and subjected to Western Blotting analysis for the acetylated H3K56, H3K14, H3K9, H4K16 and H4K5. (C) HAT, and not HDAC, are implicated in carnosol-induced histone hypoacetylation. Trichostatin A (TSA) has no influence on carnosol-induced histone H3K(56) hypoacetylation, one of p300 substrates. Cells were pre-treated with or without TSA (400 nM) for 4 hours and then treated with carnosol (50 and 100 µM) for 24 hours. Whole cell lysate was resolved on 15% SDS gel and probed with acetyl-H3K56 antibody. Data shown are representative of three independent experiments. carried at different times using different protein preparations. Bands intensities were quantified using the ImageJ software (National Institute of Health, USA).

### 3.3.1.2 Carnosol Downregulates p300 and PCAF in MDA-MB-231

Next, the protein expression level of p300, PCAF, GCN5, and hMOF histone acetyltransferases were examined by Western Blot. Carnosol was found to induce a dramatic decrease in the protein level of p300 and PCAF in MDA-MB-231 (Figure 15A) and Hs578T (Figure 15E) cells in a concentration-dependent manner. On the other hand, the protein level of GCN5 and hMOF remained constant in carnosol-treated cells. As expected, carnosol did not affect the expression level of HDAc1 and HDAC, as shown in Figure 15A. The previous results indicate that carnosol specifically downregulates p300 and PCAF in breast cancer cells.

To understand the mechanism by which carnosol downregulated p300 and PCAF, first, an examination of p300 and PCAF mRNA transcript level in MDA-MB-231 cells

treated with and without carnosol was carried out. RT-PCR showed no significant alterations in mRNA levels for both transcripts between control and carnosol-treated cells (Figure 15BAfterward, the author sought to assess whether carnosol influences p300 and PCAF constancy. To answer this question, cells were first pre-treated with cycloheximide, an inhibitor of eukaryotic translation, and after that treated with carnosol. As shown in Figure 15D, carnosol caused a decrease in the expression level of p300 and PCAF in the presence of cycloheximide. Altogether, these results strongly suggest that carnosol downregulated p300 and PCAF protein levels at the post-translational level, probably via targeting them for degradation. Additionally, the author hypothesized that histone hypoacetylation in response to carnosol is a consequence, at least partly, of p300 and PCAF downregulation.

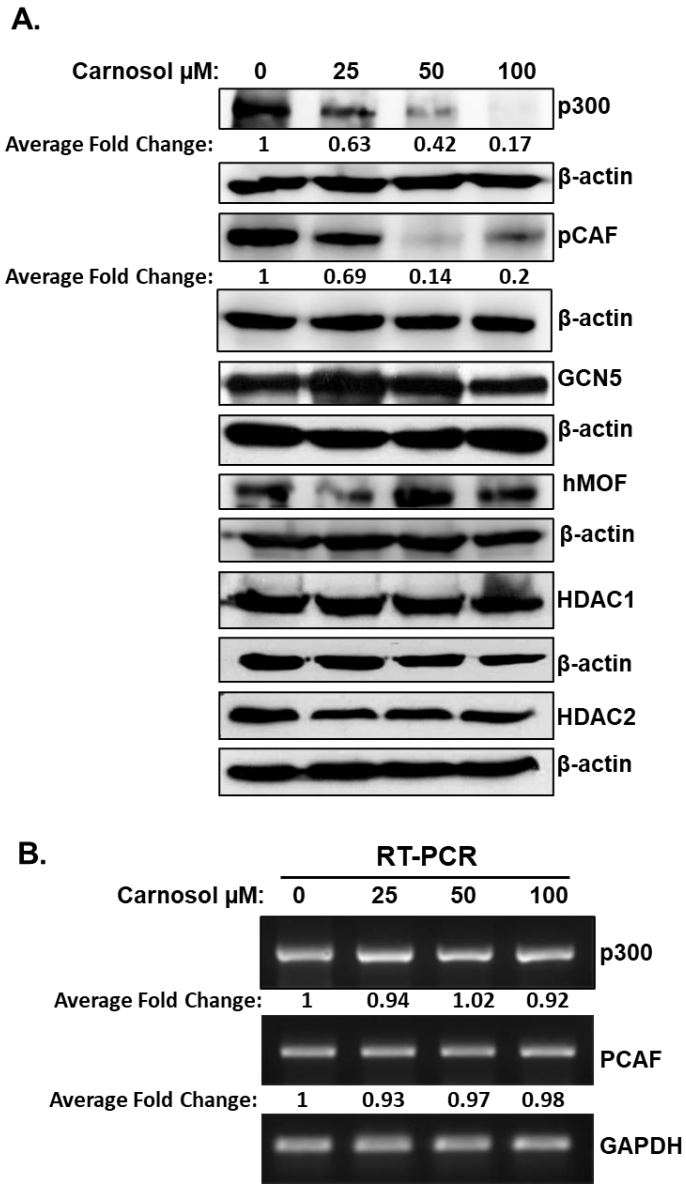


Figure 15: Carnosol downregulates the expression of p300 and PCAF in breast cancer cells.

(A) P300 and PCAF protein levels were decreased in carnosol-treated MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle (0.2% DMSO) or indicated concentrations of carnosol (50 µM and 100 µM) and whole cell lysates were resolved in 6% SDS-PAGE and subjected to Western Blot analysis for the indicated proteins. The Data shown are illustrative of three independent experiments. (B, and C) Carnosol does not affect the mRNA transcript levels of p300 and PCAF. Total RNA from cells treated with carnosol or vehicle were used to amplify the PCAF and p300 transcripts by RT- and qRT-PCR. RT- and qRT-PCR were repeated two times. (D) Carnosol downregulates p300 and PCAF proteins in cycloheximide-pre-treated cells. Cells were pre-treated with cycloheximide (an inhibitor of *de novo* protein synthesis) for 2 hours and, then treated with or without carnosol (50 µM and 100 µM). Whole cell lysates were collected and exposed to Western Blot for p300 and PCAF. (E) Downregulation of p300 and PCAF proteins expression in Hs578T cells treated with carnosol. Hs578T cells were treated with vehicle (0.2% DMSO) or indicated concentrations of carnosol (50 µM and 100 µM) and whole cell lysates were resolved in 6% SDS-PAGE and subjected to Western Blot analysis for P300 and PCAF proteins. Bands intensities were quantified using the ImageJ software (National Institute of Health, USA).

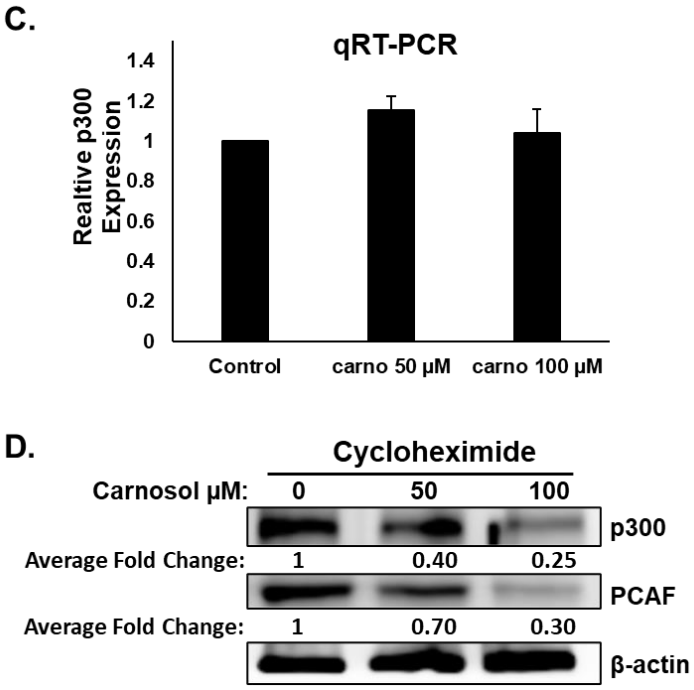


Figure 15: Carnosol downregulates the expression of p300 and PCAF in breast cancer cells (Continued).

(A) P300 and PCAF protein levels were decreased in carnosol-treated MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle (0.2% DMSO) or indicated concentrations of carnosol (50 µM and 100 µM) and whole cell lysates were resolved in 6% SDS-PAGE and subjected to Western Blot analysis for the indicated proteins. The Data shown are illustrative of three independent experiments. (B, and C) Carnosol does not affect the mRNA transcript levels of p300 and PCAF. Total RNA from cells treated with carnosol or vehicle were used to amplify the PCAF and p300 transcripts by RT- and qRT-PCR. RT- and qRT-PCR were repeated two times. (D) Carnosol downregulates p300 and PCAF proteins in cycloheximide-pre-treated cells. Cells were pre-treated with cycloheximide (an inhibitor of *de novo* protein synthesis) for 2 hours and, then treated with or without carnosol (50 µM and 100 µM). Whole cell lysates were collected and exposed to Western Blot for p300 and PCAF. (E) Downregulation of p300 and PCAF proteins expression in Hs578T cells treated with carnosol. Hs578T cells were treated with vehicle (0.2% DMSO) or indicated concentrations of carnosol (50 µM and 100 µM) and whole cell lysates were resolved in 6% SDS-PAGE and subjected to Western Blot analysis for P300 and PCAF proteins. Bands intensities were quantified using the ImageJ software (National Institute of Health, USA).

#### 3.3.1.3 Carnosol Induces ROS-Dependent Proteasome Degradation of p300 and PCAF

Al Dhaheri *et al.* have previously reported that carnosol triggered autophagy in MDA-MB-231 breast cancer cells [50]. Therefore, the author decided to explore whether p300

and PCAF were degraded through autophagy. It has been found that the blockade of early-stage autophagy (autophagosome formation) by 3-MA and late-stage autophagy (autophagolysosome formation) by CQ failed to rescue p300 and PCAF protein levels (Figure 16A). Thus, this led the author to exclude the possibility of autophagolysosomal degradation as a mechanism responsible for the decrease of the two HATs.

Afterward, the author had tested the possible involvement of proteasomal activity in a carnosol-mediated decrease of both p300 and PCAF. Firstly, MDA-MB-231 cells were pre-treated with the proteasome inhibitor MG-132 or bortezomib for 1 h before carnosol was added. As shown in Figure 16B, both proteasome inhibitors were able efficiently to restore the levels of p300 and PCAF proteins to levels comparable to untreated cells. This result clearly revealed that carnosol targets p300 and PCAF to proteasome degradation. Recently, Alsamri *et al.* have reported that carnosol stimulated proteasome degradation of STAT3 protein through a ROS-dependent mechanism [323]. This encouraged the author to check whether ROS production implicates the degradation of p300 and PCAF. Blockade of ROS accumulation using NAC, a ROS scavenger, was able to efficiently prevent carnosol-mediated proteasome degradation of the two HATs in both MDA-MB-231 (Figure 16C) and Hs578T (Figure 16F) cells. Altogether, these outcomes demonstrate that carnosol targets p300 and PCAF to ROS-dependent proteasome degradation.

Having demonstrated that proteasome inhibition (Figure 16B) and blockade of ROS accumulation (Figure 16C) efficiently restored the levels of p300 and PCAF to those observed in the untreated control cells, the author next sought to examine whether this was also associated with restoration of the histone acetylation status. Interestingly, it

has been found that the inhibition of HAT degradation by proteasome inhibitors (Figure 16D) or ROS scavenger (Figure 16E) failed to restore the histone acetylation of H3K56, a preferred p300 substrate, in MDA-MB-231. Also, NAC had no effect on carnosol-induced hypoacetylation of H3K56 in Hs578T cells (Figure 16G). Overall, these data strongly suggested that, in addition to promoting p300 and PCAF degradation, carnosol might also exert its anti-breast cancer effect via direct inhibition of HAT activity.

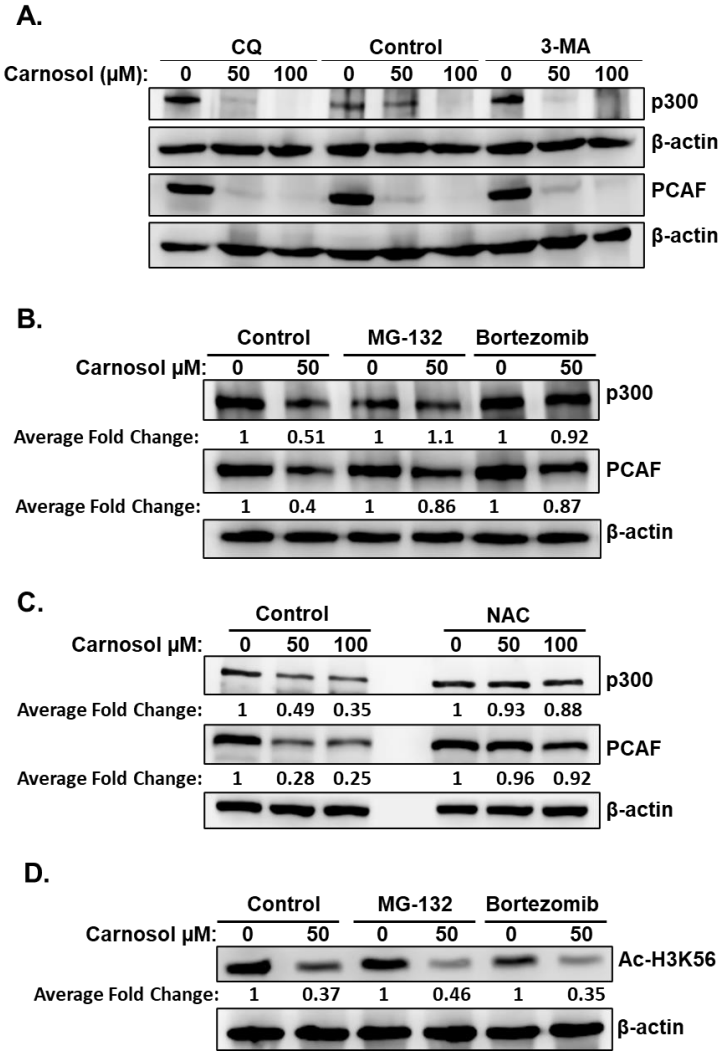


Figure 16: Carnosol stimulates a ROS-dependent proteasome degradation of p300 and PCAF in breast cancer cells.

(A) The protein expression of p300 and PCAF proteins levels in MDA-MB-231 cells pre-treated with autophagy inhibitors. Cells were pretreated with or without autophagy inhibitors (50 mM of 3-MA and 50 μM of CQ) for 1 hour and then 50 μM and 100 μM of carnosol was added and incubation for another 24 hours. (B) Carnosol causes proteasome-dependent degradation of p300 and PCAF. MDA-MB-231 were pre-treated for 1 hour with or without the proteasome inhibitors (15 μM of MG-132 or 25 nM of Bortezomib), then indicated concentrations of carnosol were applied. (C) Proteasome degradation of p300 and PCAF in a ROS-dependent manner. MDA-MB-231 cells were pre-treated for 1 hour with the ROS scavenger, NAC, then carnosol (50 and 100 μM) was added. Whole cells lysates were resolved on 6% SDS-PAGE and evaluated by Western Blotting for p300 and PCAF proteins. (D, E) Restore of p300 and PCAF expression, by proteasome inhibitors (D) and ROS scavenger (E) did not rescue the acetylation of H3K56, a preferred p300 substrate. (F) Carnosol stimulates a ROS-dependent degradation of PCAF and P300 in Hs578T cells. Hs578T cells were pre-treated for 1 hour with the ROS scavenger, NAC, then carnosol (50 and 100 μM) was added. Whole cells lysates were resolved on 6% SDS-PAGE and evaluated by Western Blotting for p300 and PCAF proteins. (G) NAC did not restore the acetylation of H3K56, a preferred p300 substrate. Whole cells lysates were resolved on 15% SDS-PAGE and evaluated by Western Blotting for acetylated H3K56. Bands intensities were quantified using the ImageJ software (National Institute of Health, USA).

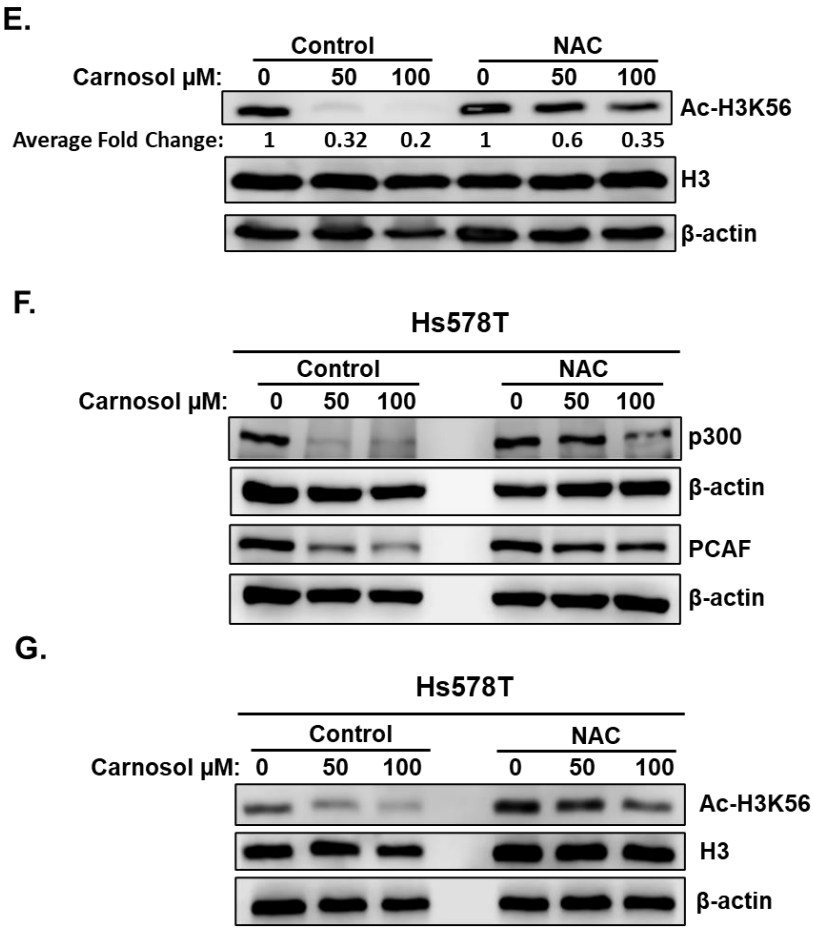


Figure 16: Carnosol stimulates a ROS-dependent proteasome degradation of p300 and PCAF in breast cancer cells (Continued).

(A) The protein expression of p300 and PCAF proteins levels in MDA-MB-231 cells pre-treated with autophagy inhibitors. Cells were pretreated with or without autophagy inhibitors (50 mM of 3-MA and 50 μM of CQ) for 1 hour and then 50 μM and 100 μM of carnosol was added and incubation for another 24 hours. (B) Carnosol causes proteasome-dependent degradation of p300 and PCAF. MDA-MB-231 were pre-treated for 1 hour with or without the proteasome inhibitors (15 μM of MG-132 or 25 nM of Bortezomib), then indicated concentrations of carnosol were applied. (C) Proteasome degradation of p300 and PCAF in a ROS-dependent manner. MDA-MB-231 cells were pre-treated for 1 hour with the ROS scavenger, NAC, then carnosol (50 and 100 μM) was added. Whole cells lysates were resolved on 6% SDS-PAGE and evaluated by Western Blotting for p300 and PCAF proteins. (D, E) Restore of p300 and PCAF expression, by proteasome inhibitors (D) and ROS scavenger (E) did not rescue the acetylation of H3K56, a preferred p300 substrate. (F) Carnosol stimulates a ROS-dependent degradation of PCAF and P300 in Hs578T cells. Hs578T cells were pre-treated for 1 hour with the ROS scavenger, NAC, then carnosol (50 and 100 μM) was added. Whole cells lysates were resolved on 6% SDS-PAGE and evaluated by Western Blotting for p300 and PCAF proteins. (G) NAC did not restore the acetylation of H3K56, a preferred p300 substrate. Whole cells lysates were resolved on 15% SDS-PAGE and evaluated by Western Blotting for acetylated H3K56. Bands intensities were quantified using the ImageJ software (National Institute of Health, USA).

### 3.3.1.4 Carnosol Inhibits p300 Acetyltransferase Activity *In Vitro*

The ability of carnosol to prevent lysine acetyltransferase activity has been tested in a cell-free system using HeLa nuclear extract (NE) as a source for HATs or recombinant p300. The results revealed that carnosol significantly inhibited HAT activity in a concentration-dependent manner in both NE- (Figure 17A) and recombinant p300-based (Figure 17F) HAT assay, suggesting that carnosol is an inhibitor of acetyltransferase activity. Afterward, the author sought to determine whether carnosol is a specific or pan-HAT inhibitor. Toward this, *in vitro* acetylation assay was used with purified histone H3 and recombinant p300, PCAF, and GCN5 acetyltransferases. Carnosol was able efficiently to inhibit p300-dependent histone acetylation (Figure 17B) while it showed no significant detectable inhibition of PCAF (Figure 17C) and GCN5-dependent histone acetylation (Figure 17D). A test for the ability of carnosol to hinder p300 HAT activity using core histones as a substrate was done. As shown in Figure 17E, carnosol also effectively inhibited the acetylation of H3K56 in this assay. Altogether, these results recognized carnosol as a new natural specific inhibitor of p300 activity.

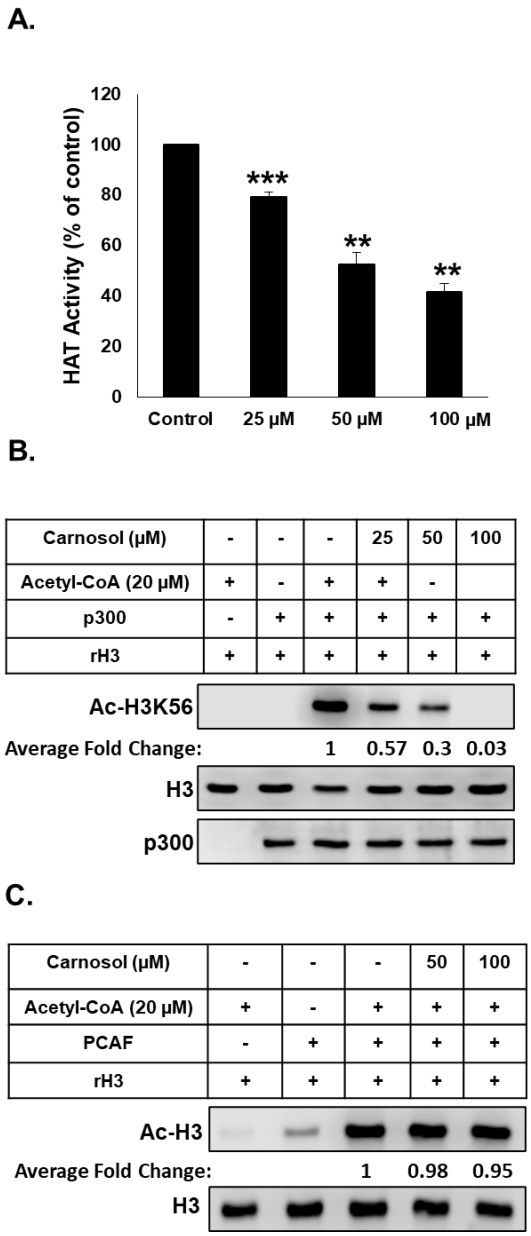


Figure 17: Carnosol is a specific inhibitor for p300 in vitro.

(A) Carnosol found to inhibit histone acetylation *in vitro*. HAT assay and using HeLa nuclear extract in the presence of increasing concentrations of carnosol (25 µM, 50 µM and 100 µM). Values represented of three independent experiments that were carried out in triplicate and presented as mean ± SEM. (**p < 0.005, ***p < 0.001). (B-D) Carnosol inhibits P300 specifically (B) but don'ts inhibits PCAF (C) or GCN5 (D). HAT assay was implemented using p300 catalytic domain (aa 1284-1673), recombinant PCAF and GCN5, recombinant H3 with or without indicated concentration of carnosol. HAT activity was evaluated by Western Blotting scoring for acetylated histone H3. (E) HAT assay was carry out using recombinant p300 catalytic domain (aa 1284-1673), core histones and with or without indicated concentrations of carnosol and HAT activity was measure as in (B). (F) In vitro HAT experiment using recombinant p300 in the present of vehicle (0.2% DMSO) and different concentrations of carnosol (25 µM, 50 µM and 100 µM). Values presented in percent in which results are calculated from three independent experiments performed in triplicate and presented as mean ± SEM. (**p < 0.005, ***p < 0.001). Bands intensities were quantified using the ImageJ software (National Institute of Health, USA).

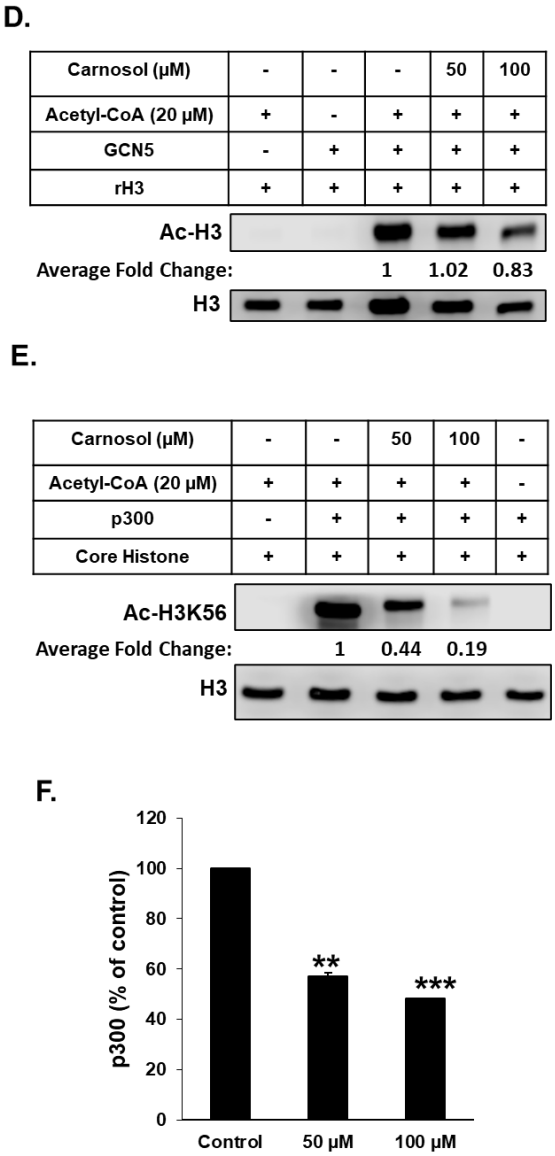


Figure 17: Carnosol is a specific inhibitor for p300 *in vitro* (Continued).

(A) Carnosol found to inhibit histone acetylation *in vitro*. HAT assay and using HeLa nuclear extract in the presence of increasing concentrations of carnosol (25 μM, 50 μM and 100 μM). Values represented of three independent experiments that were carried out in triplicate and presented as mean ± SEM. (**p < 0.005, ***p < 0.001). (B-D) Carnosol inhibits P300 specifically (B) but don'ts inhibits PCAF (C) or GCN5 (D). HAT assay was implemented using p300 catalytic domain (aa 1284-1673), recombinant PCAF and GCN5, recombinant H3 with or without indicated concentration of carnosol. HAT activity was evaluated by Western Blotting scoring for acetylated histone H3. (E) HAT assay was carry out using recombinant p300 catalytic domain (aa 1284-1673), core histones and with or without indicated concentrations of carnosol and HAT activity was measure as in (B). (F) In vitro HAT experiment using recombinant p300 in the present of vehicle (0.2% DMSO) and different concentrations of carnosol (25 μM, 50 μM and 100 μM). Values presented in percent in which results are calculated from three independent experiments performed in triplicate and presented as mean ± SEM. (**p < 0.005, ***p < 0.001). Bands intensities were quantified using the ImageJ software (National Institute of Health, USA).

#### 3.3.1.5 Carnosol Inhibits p300 Acetyltransferase Activity by Directly Competing with Acetyl CoA *In Vitro*

A collaborative work showed that *in silico* molecular docking studies that carnosol inhibits the acetyltransferase activity of p300 by hindering the entrance of the acetyl-CoA binding pocket of the catalytic domain [324].

To further confirm the *in silico* findings, *in vitro* HAT assay with carnosol, recombinant histone H3, and increasing concentrations of acetyl-CoA were carried out. As shown in Figure 16, increasing the concentration of acetyl-CoA up to 400 μM led to restoring the acetylation of H3K56 by p300. These data, alongside the molecular docking data, validates that carnosol inhibits p300 acetyltransferase activity by directly competing with acetyl-CoA binding within the acetyl-CoA binding pocket of the catalytic domain of p300.

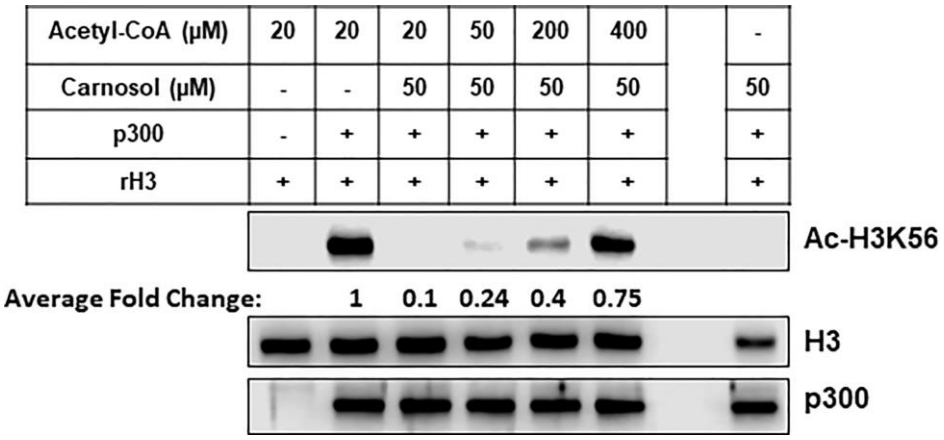


Figure 18: Carnosol competes with acetyl-CoA in the binding to the P300 catalytic domain.

Recombinant p300 catalytic domain (aa 1284-1673) was incubated with recombinant histone H3 and increasing concentrations of acetyl-CoA in the presence of 50 µM carnosol. Histone acetylation was monitored by Western blotting scoring of acetylated H3K56. Data shown are representative of three independent experiments. Bands intensities were quantified using the ImageJ software (National Institute of Health, USA).

### 3.3.2 Discussion

Here, in the third section of the study, carnosol, a natural polyphenol and major constituent of sage, rosemary, and oregano have been found to induce histone hypoacetylation in breast cancer cells, MDA-MB-1231 and Hs578T. In addition, carnosol constrained the activity of p300 acetyltransferase *in vitro* and stimulated its ROS-dependent proteasome degradation along with PCAF acetyltransferase. Lastly, carnosol is reported to hinders the activity of p300 acetyltransferase by obstructing the binding of acetyl-CoA to its pocket in the catalytic domain.

The histone acetyltransferase p300 is also known as a transcriptional coactivator and consists of several conserved domains involving acetyl-lysine binding bromodomain, zinc-binding domain, and a centrally located HAT domain. These domains directly interact with various cellular proteins and transcriptional factors [280]. The p300 HAT

domain is organized as a central $\beta$-sheet containing seven $\beta$-strands surrounded by loops and nine $\alpha$-helices. This HAT domain can be further split into two subdomains, a smaller C subdomain that comprises three $\alpha$-helices and one $\beta$-strand that spans the entire structure by covering the opposite ends of the larger N subdomain [325]. The acetyl-CoA binding site of p300 resides located in a deep hydrophobic cavity that extends into the interior of the protein. The back of the hydrophobic cavity is lined by residues Ile1395, Tyr1397, Leu1398, and Ile1435. The larger number of interactions and better binding affinity of acetyl-CoA could be attributed to its size. Among HATs, the presence of an unusually long substrate-binding loop, L1, located between $\alpha$4 and $\beta$5 is a feature unique to p300 and appears to encapsulate acetyl-CoA. In all the acetyl-CoA structures of p300, Arg1410 bounded to the phosphates of acetyl-CoA by a hydrogen bond. The significance of this interaction has been established by mutagenesis and inhibition studies using 3′-dephospho-Lys-CoA [326]. Carnosol has been found to occupy the same region where the pantetheine arm of acetyl-CoA is bound. Both the pantetheine arm and lactone moiety made extensive interactions with L1, the long loop. This kind of extensive interaction sequentially permits the L1 loop to interact intramolecularly with other regions in p300's HAT domain [327]. Carnosol would fight with the incoming substrate in order to position it in the substrate-binding grove of p300. In the substrate-binding loop, lysine residue contributed to the most energetic interaction in the p300-carnosol complex. Altering this to alanine did not cause any noteworthy change in the binding site and energy, demonstrating the importance of the contribution of the whole loop in the binding of carnosol in p300. Finally, carnosol binds to p300 with better binding energy than the known p300-specific inhibitor C646 and the HAT inhibitor CPTH2.

It is well reported that p300 functions as a coactivator of multiple transcription factors to regulate the expression of many genes that control many biological processes [328,329]. Also, the HAT activity of p300, which is weak intrinsically, is stimulated by the autoacetylation of some residues located in the HAT domain [330]. Here, the third section of the study has shown that in addition to targeting p300 to degradation, carnosol could inhibit the acetyltransferase activity required for autoacetylation and acetylation of target proteins. The *in silico* molecular docking data and the competition assay suggested that carnosol inhibits p300 activity by blocking the entry of acetyl-CoA into the active site and hence its autoacetylation, which further confirmed by *in silico* molecular docking carried by Dr. Ranjit Vijayan. Several observations suggest that the HAT activity of p300 is essential for its stability. Work by Jain and collaborators revealed that autoacetylation is also crucial for p300 stabilization during oxidative stress [331], thus autoacetylation of p300 not only stimulates p300 transactivation function but also contributes to its stabilization. Indeed, these studies showed that treating rat cardiac myocytes with ROS inducer, doxorubicin, led to p300 stabilization. Remarkably, the stabilization of p300, which reduces its resistance to proteasome-degradation, was entirely due to an increase in the acetylation of p300 that was dependent on its HAT activity. The half-life of p300 was reduced in the presence of the inhibitor of p300, anacardic acid [331]. Also, curcumin, known to inhibit the HAT activity of p300 selectively, induced oxidative stress and was reported to stimulate the proteasomal-dependent degradation of p300 [241]. Based on these observations, it becomes tempting to hypothesize that the lack of p300 autoacetylation, due to the inhibition of HAT activity, combined with oxidative stress could account, although possibly not solely, for the targeting of p300 protein to proteasomal degradation. Al Dhaheri *et al*. and Alsamri *et al*. have previously reported that carnosol induced a

dramatic generation of ROS in triple-negative breast cancer cells, Hs578T and MDA-MB-23 [50,323].

Here, section 3 of the study reported that carnosol inhibited p300 HAT activity and promoted its proteasome degradation thru ROS generation. Additionally, it has been showen that blockade of the proteasomal activity or ROS generation restored p300 protein that lacks HAT activity. Indeed, the rescue of p300 was not connected with the restoration of acetylation of its preferred substrate, H3K56. Altogether, these results support the hypothesis that one possible mechanism through which carnosol targets p300 to degradation comprises the inhibition of p300 autoacetylation combined with induction of oxidative stress in breast cancer cells.

An increasing body of evidence implies that p300 is a positive regulator of cancer progression and is associated with tumorigenesis of various human cancers, including the invasive MDA-MB-231 breast cancer [216,218,332–334]. It has been shown that Knockdown of p300 reduced proliferation and caused a rise of caspase-dependent apoptosis of both intrinsic and extrinsic apoptotic pathways in prostate cancer cells [201]. Moreover, the cellular invasion was decreased upon p300 depletion, which reduced MMP-9 transcription [201]. Kim and coworkers exhibited that MMP-9 activation requires p300 and LYR71, a derivative of trimeric resveratrol, to reduce MMP-9 transcripts via blocking p300 recruitment and deacetylating histone H3 and H4 on the MMP-9 promoter [249]. Interestingly, Alsamri *et al.* showed that carnosol inhibited the potential invasion of MDA-MB-231 by downregulating MMP-9 expression, targeting the transcription factor STAT3 to proteasome degradation [323]. Herein, section 3 showed that carnosol dramatically reduced the level of p300 in MDA-MB-231 breast cancer cells. Thus, p300 degradation may contribute to the

downregulation of MMP9 and, therefore, inhibition of invasion of MDA-MB-231 cells.

The role of PCAF in tumorigenesis is somewhat controversial and seems to depend on the type of cancer. PCAF was reported to function as a tumor suppressor in the case of Hepatocellular carcinoma [230,335], gastric cancer [336], lung adenocarcinoma [337(p. 6)] and colorectal cancer [338] while, in other cancers such as medulloblastoma and glioblastoma, it contributes to cytoprotective events that help cancer cells to survive, evade executing cell death programs and invade other tissues [339]. However, there are only a few studies carried out on the role of PCAF in breast cancer. PCAF-mediated acetylation at H3K9 at the multidrug resistance (MDR) genes was shown to contribute to the multidrug resistance of breast cancer and, Knockdown of PCAF significantly sensitizes these cancer cells to anticancer therapeutics [215]. Park *et al.* [340] showed that pharmacological inhibition of PCAF by N-Acylanthranilic Acids [340]. Recently, Song *et al*. showed that Acetylation of ARF6 GTPase-activating protein ACAP4 at Lys311 by PCAF is essential for the CCL18-elicited cell migration and invasion in MDA-MB-231and MDA-MB-468 cancer breast cancer cells. Inhibition of PCAF activity by C146 (PCA inhibitor) abolished the acetylation of ACAP4 and consequently impaired the migratory and invasive ability of MDA-MB-231 cells. Similar inhibition was observed in PCAF-depleted MDA-MB-231 cells [341]. The inhibition of both p300 and PCAF acetyltransferases activity was also shown to exert an anti-breast cancer effect. Indeed, anacardic acid, an inhibitor of PCAF and p300 activity [236(p. 3)], induced cell cycle arrest and apoptotic cell death in MDA-MB-231 cells [342]. In addition, it was also reported that anacardic acid efficiently and significantly inhibited cell migration and invasion and downregulated the expression of MMP-9 in MDA-MB-231 cells [342,343].

Here, the results revealed for the first time that carnosol targets PCAF to ROS-dependent proteasome degradation in breast cancer cells, which contribute to its anticancer activity. The molecular mechanism by which PCAF is targeted to degradation is yet to be uncovered. In agreement with the previous findings, our data strongly suggest that PCAF contributes to breast cancer survival and/or metastasis and represents a rational target for breast cancer therapy.

# Chapter 4: Conclusion

In this study, we investigated the efficacy of carnosol to inhibit breast cancer *in vitro* and *in vivo*. Carnosol inhibited tumor growth derived from the triple-negative breast cancer cells, MDA-MB-231. In addition, it significantly inhibited invasion and metastasis via downregulating the activity and expression of MMP-9, inhibiting STAT3 signaling pathway through a ROS-dependent proteasome degradation of STAT3 protein. Moreover, carnosol has been shown to induce ROS-dependent ER stress through upregulating the three main UPR sensors and their downstream targets which includes ATF4/CHOP, ATF6α, and IRE1α/XBP1. Furthermore, we showed that carnosol upregulated β-catenin while downregulating both Akt and mTOR. These later proteins were targeted to ROS-dependent proteasome degradation. Interestingly, we found carnosol-induced ER stress is dependent upon upregulation of the p38 MAPK signaling pathway. Chemical inhibition of p38 dramatically reduced the level of CHOP in carnosol-treated cells hence demonstrating the involvement of this pathway in the induction of ER stress. A biochemical approach showed for the first time that carnosol is a direct and selective inhibitor of the p300 histone acetyltransferase whose activity was shown to mediate cancer invasion and metastasis. Finally, it is worth mentioning that carnosol is a pharmacologically safe compound with no side effects observed in different mice models. We propose the following model (Figure 19) to summarize the mechanisms through which carnosol mediates anticancer activities against the triple negative breast cancer.

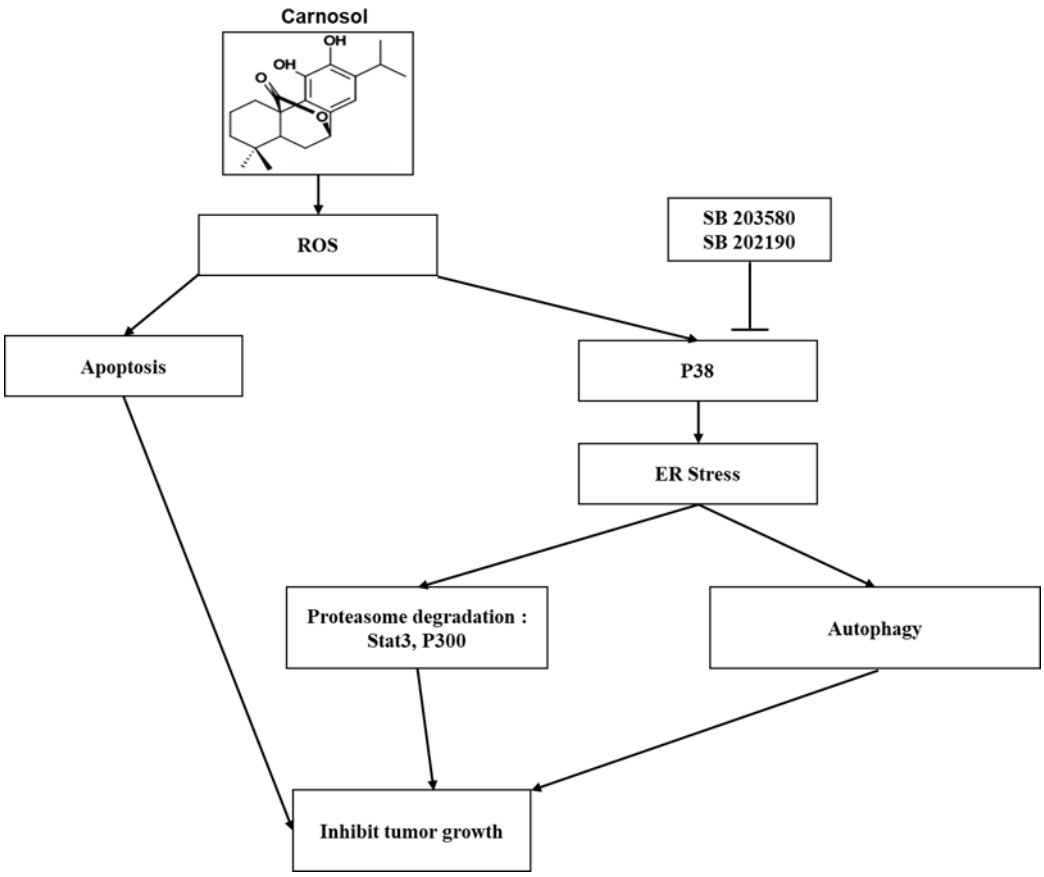


Figure 19: A hypothesized schematic model summarizing the possible mechanism of action carnosol has in TNBC.

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## List of Publications

1. Alsamri, H., Hasasna, H. E., Baby, B., Alneyadi, A., Dhaheri, Y. A., Ayoub, M. A., Eid, A. H., Vijayan, R., & Iratni, R. (2021). Carnosol Is a Novel Inhibitor of p300 Acetyltransferase in Breast Cancer. *Frontiers in Oncology, 11*, 664403. https://doi.org/10.3389/fonc.2021.664403

2. Alsamri, H., Athamneh, K., Pintus, G., Eid, A. H., & Iratni, R. (2021). Pharmacological and Antioxidant Activities of Rhus coriaria L. (Sumac). *Antioxidants (Basel, Switzerland), 10*(1). https://doi.org/10.3390/antiox10010073

3. Athamneh, K., Alneyadi, A., Alsamri, H., Alrashedi, A., Palakott, A., El-Tarabily, K. A., Eid, A. H., Al Dhaheri, Y., & Iratni, R. (2020). Origanum majorana Essential Oil Triggers p38 MAPK-Mediated Protective Autophagy, Apoptosis, and Caspase-Dependent Cleavage of P70S6K in Colorectal Cancer Cells. *Biomolecules, 10*(3), E412. https://doi.org/10.3390/biom10030412

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5. Alsamri, H., El Hasasna, H., Al Dhaheri, Y., Eid, A. H., Attoub, S., & Iratni, R. (2019). Carnosol, a Natural Polyphenol, Inhibits Migration, Metastasis, and Tumor Growth of Breast Cancer via a ROS-Dependent Proteasome Degradation of STAT3. *Frontiers in Oncology, 9*, 743. https://doi.org/10.3389/fonc.2019.00743

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8. El Hasasna, H., Saleh, A., Al Samri, H., Athamneh, K., Attoub, S., Arafat, K., Benhalilou, N., Alyan, S., Viallet, J., Al Dhaheri, Y., Eid, A., & Iratni, R. (2016). Rhus coriaria suppresses angiogenesis, metastasis and tumor growth of breast cancer through inhibition of STAT3, NFκB and nitric oxide pathways. *Scientific Reports, 6*, 21144. https://doi.org/10.1038/srep21144

9. El Hasasna, H., Athamneh, K., Al Samri, H., Karuvantevida, N., Al Dhaheri, Y., Hisaindee, S., Ramadan, G., Al Tamimi, N., AbuQamar, S., Eid, A., & Iratni, R. (2015). Rhus coriaria induces senescence and autophagic cell death in breast cancer cells through a mechanism involving p38 and ERK1/2 activation. *Scientific Reports, 5*, 13013. https://doi.org/10.1038/srep13013

# Appendix

| Cell lines/ Reagent / Antibodies / rProteins | Catalog No. | Manufacturer |
|---|---|---|
| MDA-MB-231 | 300275 | Cell Line Service (CLS)-GmbH, Germany |
| Hs578T | HTB-126 | ATCC-USA |
| MCF7 | HTB-22 | ATCC-USA |
| T47D | 300353 | Cell Line Service (CLS)-GmbH, Germany |
| HCT116 | CCL-247 | ATCC-USA |
| DMEM, high glucose, pyruvate | 41966029 | Gibco™, Thermo Scientific, UK |
| RPMI 1640 Medium | 21875034 | Gibco™, Thermo Scientific, UK |
| Fetal Bovine Serum (FBS), qualified, Brazil | 10270106 | Gibco™, Thermo Scientific, UK |
| Penicillin-Streptomycin-Glutamine (100X) | 10378016 | Gibco™, Thermo Scientific, UK |
| DPBS (1X) | 14190094 | Gibco™, Thermo Scientific |
| Trypsin-EDTA (0.25%), phenol red | 25200056 | Gibco, Life Technologies, Rockville, UK |
| Carnosol | C9617-5MG | Sigma Aldrich, US |
| Chloroquine diphosphate | ab142116 | Abcam |
| Autophagy Inhibitor, 3-MA | CAS 5142-23-4 | Sigma-Aldrich |
| MG-132 | 2194 | Cell Signalling |
| Bortezomib | 2204 | Cell Signalling |
| Caspase Inhibitor I (Z-VAD-FMK) | 187389-52-2 | Sigma Aldrich, US |
| N-Acetyl-L-cysteine (NAC) | 616-91-1 | Millipore |
| Trichostatin A (TSA) | 9950S | Enzo |
| goat anti-mouse IgG-HRP | sc-2005 | Santa Cruz Biotechnology |
| goat anti-rabbit IgG-HRP | sc-2004 | Santa Cruz Biotechnology |
| β-Actin Antibody (C4) | sc-47778 | Santa Cruz Biotechnology |
| Phospho-Stat3 (Tyr705) | 9131 | Cell Signalling |
| Stat3 (124H6) Mouse | 9139 | Cell Signalling |
| Anti-Histone H3 antibody [EPR17785] | ab201456 | Abcam |
| Acetyl-Histone H3 (Lys9/Lys14) | 9677 | Cell Signaling |
| Anti-Histone H4, Acetylated (2-19) Rabbit pAb | 382160 | Sigma Aldrich, US |
| Anti-Histone H4 | 07-108 | Sigma Aldrich, US |

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| Anti-Histone H3 (acetyl K56) antibody [EPR996Y] | ab76307 | Abcam |
| Acetylated-Lysine | 9441 | Cell Signaling |
| Acetyl-Histone H3 (Lys14) (D4B9) Rabbit | 7627 | Cell Signaling |
| Acetyl-Histone H3 (Lys9) (C5B11) Rabbit | 9649 | Cell Signaling |
| Anti-Histone H4 (acetyl K16) antibody [EPR1004] | ab109463 | Abcam |
| Acetyl-Histone H3 (Lys14) (D4B9) | 7627 | Cell Signaling |
| Acetyl-Histone H3 (Lys9) (C5B11) Rabbit mAb | 9649 | Cell Signaling |
| Acetyl-Histone H4 (Lys5) | 9672 | Cell Signaling |
| Anti-Histone H4 Antibody | 07-108 | Sigma Aldrich |
| Anti-Histone H4, Acetylated (2-19) | 382160 | Sigma Aldrich |
| p300 Antibody (F-4) | sc-48343 | Santa Cruz Biotechnology |
| Acetyl-CBP (Lys1535)/p300 (Lys1499) | 4771 | Cell Signaling |
| PCAF Antibody (E-8) | sc-13124 | Santa Cruz Biotechnology |
| GCN5 Antibody (H-75) | sc-20698 | Santa Cruz Biotechnology |
| Anti-acetyl-Histone H3 | 06-599 | Sigma Aldrich |
| HDAC1 Antibody (H-51) | sc-7872 | Santa Cruz Biotechnology |
| HDAC2 Antibody (C-8) | sc-9959 | Santa Cruz Biotechnology |
| GAPDH Antibody (FL-335) | sc-25778 | Santa Cruz Biotechnology |
| Recombinant Human STAT3 protein | ab43618 | Abcam |
| p300 (catalytic domain) (human), (recombinant) | BML-SE451-0100 | Enzo |
| Recombinant human Histone H3 protein | ab198757 | Abcam |
| CREB binding protein (catalytic domain) (human), (recombinant) | BML-SE452-0100 | Enzo |
| GCN5 (human), (recombinant) | BML-SE272-0050 | Enzo |
| pCAF / Histone H3 (human) (5-23) amide | BML-P271-0500 | Enzo |
| Acetyl coenzyme A sodium salt, 5 mg | sc-210745A | Santa Cruz Biotechnology |
| Core Histone Proteins | 13-107 | Sigma Aldrich |
| PMSF | 36978 | Thermo scientific |
| 40% Acrylamide/Bis 29:1 500ml | HC2040 | Thermo Scientific |
| TEMED | 17919 | Thermo Scientific |
| APS (Ammonium Persulfate) | 17874 | Thermo Scientific |

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| PageRuler Plus Prestained Protein Lader | 26619 | Thermo Scientific |
| Western Blot Stripping buffer 500ml | 21059 | Thermo Scientific |
| 10x Tris-Glycine Buffer | 28363 | Thermo Scientific |
| Sodium Dodecyl Sulfate SDS | sc-264510c | chem cruz |
| Trizma Base | T1503-1KG | SIGMA ALDRICH |
| Glycine | G8898-1 KG | SIGMA ALDRICH |
| Mercaptoethanol | M6250-10ML | SIGMA ALDRICH |
| Tween™ 20 | 28320 | Thermo Scientific™ |