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EVALUATION OF THE EFFECTS OF WALNUT EXTRACT ON CELL DIVISION
G2/M CYCLIN - CYCLIN B1 IN BREAST CANCER CELLS

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Savita Shah

May 2016

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The Designated Thesis Committee Approves the Thesis Titled

EVALUATION OF THE EFFECTS OF WALNUT EXTRACT ON CELL DIVISION
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by

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ABSTRACT

EVALUATION OF THE EFFECTS OF WALNUT EXTRACT ON CELL DIVISION G2/M CYCLIN - CYCLIN B1 IN BREAST CANCER CELLS

by Savita Shah

Walnuts are rich in polyphenols and have potential for cancer prevention and treatment. Our lab has previously shown that a walnut extract (WE) is able to block the cell cycle at the S and G2 phases of cell division with a corresponding decrease in the amount of the cyclin B1 protein and induce cell death in human breast cancer cells (Le et al., 2014). The aim of the current research was to identify whether this effect on the expression of cyclin B1 was at the transcriptional or the translational level. A cycloheximide (CHX) chase revealed that a WE does not affect the half-life of cyclin B1. A time course analysis in conjunction with real-time quantitative polymerase chain reaction (RT-qPCR) revealed that a WE decreased the number of cyclin B1 transcripts. A similar effect was observed with other cyclins, cell proliferation marker Ki67, and the transcription factor Sp1. These results suggested that WE affect the expression of cyclins and genes involved in cell proliferation. Further, there was no significant decrease in the levels of mRNA of Notch1, RelA/p65, and MXD4. We also confirmed that repression of the cyclin B1 gene was dose dependent and was specific to the ellagitannins, tellimagrandin I and casuarictin, isolated from walnuts. Quercetin, a flavanol also found in walnuts, did not have any effect on cyclin B1 expression. Overall, our results suggest that in WE-treated MDA-MB-231, a decrease in the amount of the cyclin B1 protein is correlated with a corresponding decrease in its transcription.

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List of Abbreviations

ANOVA:	analysis of variance
APC:	anaphase promoting complex
ATCC:	American type culture collection
ATM:	Ataxia Telangiectasia Mutated
ATR:	Ataxia Telangiectasia and Rad3 Related
BA:	butyric acid
CAK:	cdk-activating kinase
cDNA:	complimentary DNA
CDC:	cell division cycle
CDK:	cyclin-dependent kinase
CHK:	checkpoint kinase
CHX:	cycloheximide
CIDEC:	cell death-inducing DFFA-like effector protein C
CIK:	inhibitor of cyclin-cyclin dependent kinase complex
CIP:	cdk interacting protein
CTD:	carboxy-terminal domain
DEPC:	diethylpyrocarbonate
DMEM:	Dulbecco's modification of Eagle's medium
DMSO:	dimethyl sulfoxide
EGF:	epidermal growth factor
EGFR:	epidermal growth factor receptor

ER: estrogen receptor

ESI-IT-TOF-MS: electrospray ionization ion trap time of flight mass spectroscopy

FBS: fetal bovine serum

G1: gap phase 1

G2: gap phase 2

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

HHDP: hexahydroxydiphenic acid

HRP: horseradish peroxidase

HSCCC: high speeds counter current chromatography

HSD: honest significant difference

IGF: insulin growth factor

IK- κ B: inhibitor of kinase κ B

JAK: Janus kinase

KIP: cyclin-dependent kinase inhibitor

LC-LTQ-Orbitrap: liquid chromatography coupled with electrospray ionization hybrid
linear trap quadrupole -Orbitrap

MAPK: mitogen activated protein kinase

MAT1: ménage a trois

MAX: Myc-associated factor X

MGA: MAX-gene associated protein

miR: microRNA

MNT: MAX-network transcriptional repressor

MSMB: microsemino protein B

mTOR: mechanistic target of rapamycin

MXD4: MAX-dimerization protein 4

NF- κ B: nuclear factor kappa B

PI(3)K: phosphatidylinositol (3) kinase

PKC: protein kinase C

PR: progesterone receptor

p-TEFB: positive transcription elongation factor b

RBI: retinoblastoma proteins

RNA pol II: RNA polymerase II

RT-qPCR: real time quantitative polymerase chain reaction

SAC: spindle assembly checkpoint

SOD: superoxide dismutase

Sp1: specificity protein 1

STAT: signal transducer and activator of transcription

TFIIH: transcription factor IIH

TNBC: triple negative breast cancer

TPA: tetradecanoylphorbol-13-acetate

TRAMP: transgenic adenocarcinoma of the mouse prostate model

VEGF: vascular endothelial growth factor

WE: walnut extract

Introduction

Cancer is one of the leading causes of death worldwide. An annual report published by the American Cancer Society has projected that in the year 2016; approximately 1,658,210 new cancer cases will be diagnosed in the US (American Cancer Society, 2016). Of the new cancer cases, 249,260 will be diagnosed as cases of invasive breast cancer, leading to approximately 40,890 deaths.

Apart from radiation and chemotherapy, there are options of targeted therapy available to patients who exhibit the presence of estrogen receptor (ER⁺), progesterone receptor (PR⁺), or human epidermal receptor 2 (Her2⁺) on the surface of breast cancer cells. Triple negative breast cancers (TNBC) are characterized by the absence of all three molecular markers (ER⁻, PR⁻, and Her2⁻). Patients with TNBC have the highest risk of recurrence and have the shortest overall survival rate compared to the patients that exhibit the presence of at least one of the three molecular markers (Bauer et al., 2007; Dent et al., 2011; Gallanina et al., 2011). Chemotherapeutic drugs like doxorubicin and paclitaxel are given to TNBC patients. However, these drugs have severe side effects and in some cases have been shown to promote tumor progression (Vassileva et al., 2008; Daenen et al., 2011). There is a need to find new alternate bioactive agents from natural sources, such as polyphenols, that can improve a patient's survival rate with few side effects.

Polyphenols are ubiquitously expressed in plants and are the most abundant antioxidants in the human diet that also display anti-inflammatory, anti-microbial, anti-viral, anti-cancer and immunomodulatory activities (Scalbert et al., 2005; Benvenuto et al.,

2013; Marzocchella et al., 2011; Izzi et al., 2012; Vallianou et al., 2015). They can inhibit cancer cell growth by interacting with multiple signal transduction pathways (e.g., NF- κ B, AKT, PI(3)K, and EGFR) that are mis-regulated in cancer (Sun et al., 2012; Parekh et al., 2011; Estrov et al., 2003; Zhao et al., 2014).

Among nuts, walnuts have the highest total phenolic content (Li et al., 2006; Abe et al., 2010; Vinson & Cai, 2012). The highest total antioxidant compounds were reported by Blomhoff and his co-workers in walnut seeds with pellicles that have a range of 10.8-33.3 mmol antioxidants/ 100 g (Blomhoff et al., 2006). Consumption of a diet rich in walnuts inhibited the initiation and progression of growth of breast cancer and prostate cancer xenografts in mice (Hardman & Ion, 2008; Davis et al., 2012; Reiter et al., 2013). A methanol extract generated from walnut leaves, husk and seeds exhibited a concentration-dependent antiproliferative effect on renal and colon cancer cells (Carvalho et al., 2010). Some researchers have reported an effect of a WE on the cell proliferation and the cell cycle of melanoma (Aithal et al., 2009; Yang et al., 2009; Carvalho et al., 2010; Salimi et al., 2012; Le et al., 2014). Previous studies performed in our lab on MDA-MB-231 cells treated with a WE indicated a time and dose- dependent effect on cell viability, cell cycle arrest at the G2 phase and a decrease in expression of the cyclin B1 protein (Le et al., 2014). These studies did not address whether this decline in cyclin B1 levels was due to inhibition of transcription or translation.

The goal of the current research was to determine whether the decrease in the expression of the cyclin B1 protein in TNBC cell line, MDA-MB-231, treated with a WE is transcriptional or translational. Since there are limited successful chemotherapeutics

available for TNBC, understanding the effect of a WE on the cell cycle related proteins at a molecular level could open an attractive possibility of combinatorial therapy of WE and conventional drug(s) in treating TNBC.

Literature Review

Overview of Cell Cycle Progression

The cell cycle is a sequence of defined changes that occur in a cell in response to mitogenic stimulation resulting in the formation of two genetically identical cells (Figure 1).

Figure 1. Eukaryotic cell cycle. The cell cycle comprises two major phases, interphase and mitosis. Interphase, the longer phase of the cell cycle, is divided into three sub-phases: G1 phase, S phase and G2 phase. The G1 phase can last up to 11 h, the S phase, or the DNA synthesis phase lasts up to 8 h, and the G2 phase lasts up to 4 h. The mitotic phase (M) is the shortest phase in the cell cycle. It is further divided into prophase, metaphase, anaphase, and telophase.

The resting phase, also known as the G0 phase, is the phase where the cells do not replicate. The cells in this phase may be quiescent (dormant) or senescent (ageing or

deteriorating). These cells remain in the G0 phase until they are stimulated by mitogenic stimuli, *i.e.*, growth factors, intercellular signals, or cytokines.

The cell cycle is divided into two distinct phases: interphase and the mitotic phase (Norbury and Nurse 1992). Interphase is further divided into three sub-phases: Gap phase 1 (G1), DNA synthesis (S), and Gap phase 2 (G2). Interphase is the longer phase of the cell cycle where the cell prepares itself for division. The G1 phase can last up to 11 h and is marked by a dominant cyclin D/cdk4/6 activity and a gradual increase in the levels of cyclin E1/cdk2 activity. The S phase can last upto 8 h, and a gradual rise in the levels of cyclin A/cdk2 protein is seen in this phase. The G2 phase can last up to 4 h and is marked by the highest concentration of the cyclin B1/cdk1 complex. The mitotic phase (M) is the shortest phase of the cell cycle and is further divided into prophase, metaphase, anaphase, and telophase. In M phase, the chromosomes separate and are distributed into two daughter cells. Following cytokinesis, the cell is successfully divided into two genetically identical cells (Norbury & Nurse, 1992).

Control of the cell cycle. Cyclin-dependent kinases (cdks) are heterodimeric proteins with kinase domains that are active only when bound to regulatory proteins called cyclins. Any change of the phase in the cell cycle is tightly regulated by the cdks. The cdks are serine/threonine kinases that are activated at specific points in the cell cycle. Their levels remain constant throughout the cell cycle (Morgan, 1995). Note that the time-varying concentrations implied in Figure 1 apply only to the cyclins and not the cdks. Out of more than 20 known cdks (Malumbres et al., 2009), activities of four cdks (cdk1, cdk2, cdk4, and cdk6) are dominant in the cell cycle.

Cyclins are a diverse group of proteins and are classified based on the structure of the cyclin-box that mediates their binding to the cdks. Cyclins can be subdivided into those that oscillate (e.g., cyclins D1, D2, D3; cyclins A1, A2; cyclin E1 and cyclins B1, B2) and, others that do not oscillate (e.g., cyclins C, H, K, T, Y, and L). The pattern of expression of cyclins varies with progression through the cell cycle. The pattern also defines the relative position of the cell within the cell cycle (Gopinathan, 2011).

Regulation of the cell cycle. The cell cycle is regulated by various processes involving a diverse, coordinated, and error-resistant regulatory mechanism that assures precise cell division. Some of the important proteins in the regulation of the cell cycle are illustrated in Figure 2. In response to a mitogenic stimulus, cells transition from G₀ into the G₁ phase of the cell cycle.

G₁ phase of the cell cycle. The transition from G₀ to G₁ commits a cell to the entire cell cycle process. The mitogenic factors activate Ras and the phosphatidylinositol-3-kinase (PI(3)K) pathway. This activation stimulates cell proliferation, growth, and survival through stabilization of a transcription factor, c-myc, or by the activation of a cellular transcription factor, NF- κ B. These transcription factors induce the expression of cyclin D as well as suppress the activity of the cyclin-dependent kinase inhibitor 2A (CDKN2A/ INK4A /p16) (Sears & Nevins, 2002).

Cyclin D associates with cdk4/6 and is activated by the the cdk-activating kinase (CAK), the cyclin H/cdk7/ MAT1 complex. The activated cyclin D/cdk4/6 complex hyper-phosphorylates tumor suppressor proteins, such as the retinoblastoma protein (RB1) and the retinoblastoma-like proteins e.g., p107 (RB11) and p130 (RB12). This results in a

release of E2F transcription factors (E2Fs). The E2Fs activate genes whose products are necessary for cell proliferation and cell division (Johnson & Schneider-Brossard, 1998; Lim & Kaldis, 2013).

Figure 2. Schematic overview of some of the important proteins in cell cycle regulation. c-Myc, AP1 and NF-κB are the transcription factors for the genes responsible for cell proliferation, CDC25 is a cyclin/cdk-activating phosphatase, pRb- is a retinoblastoma protein, the INK4 family (p15, p6, p18, and p19) are the inhibitors of cdk4, CIP/KIP family of proteins (p21, p27, and p57) are the inhibitors of Cyclin/cdk2/4 complexes, 14-3-3 proteins inactivate CDC25, Wee-1 is a cyclin B1/cdk1 inactivating kinase. CHK1 and CHK2 are the two checkpoint kinases. TGF-β is a tumor growth factor β. APC-CDC20 is a protein complex comprising of an anaphase-promoting complex and the cell division cycle 20 protein.

Towards the end of the G1 phase, CDC25A/B/C activates the cyclin E/cdk2 complex. This complex hyperphosphorylates and releases more E2Fs from the RB1, RB11, and RB12 complexes. The E2Fs activate other cell cycle-related genes whose products are

important for DNA synthesis in the S phase (Weinberg, 1995; Harbour & Dean, 2000). Cyclin E1/cdk2 also phosphorylates histone H1, which is important for the chromatin rearrangements required in the S phase of the cell cycle (Harbour & Dean, 2000). In the G1 and S phases, the cyclin E1/cdk2 complex also phosphorylates the cyclin/cdk2/4 inhibitors p27 and p21, thereby keeping cyclin D/cdk4/6 and cyclin A1/cdk2 complexes active during the G1 and S phases of cell cycle, respectively. At the end of the G1 phase, the cyclin D/cdk4/6 complex is inactivated either by the inhibitors of cdk4/6 (the CDKN/INK4 family of inhibitors, e.g., p16, p16, p18, and p19), or by the inhibitors of cyclin-cdk 2/4 (the CIP/KIP family of inhibitors, e.g., p21, p27, and p57) (Sherr & Roberts, 1999).

Prior to entering the S phase, the cell reaches a G1/S checkpoint that allows it to fix any defects that may have occurred during the G1 phase. In the event of single-strand DNA breaks, ataxia telangiectasia and rad3-related (ATR) protein kinase, a serine/threonine kinase, phosphorylates checkpoint kinase 1 (Chk1). Chk1 arrests the cell at the G1 phase until the damage has been repaired (Bartek & Lukas, 2003). To stop the activation of cyclinE/cdk2 complex, a CDC25 phosphatase family of proteins is degraded until all damage is repaired. After crossing this checkpoint, the cell is committed to cell division and cannot return back to the G0 phase.

S phase of the cell cycle. DNA synthesis/replication occurs in the S phase of the cell cycle. In this phase, cyclin A is seen co-localized with the DNA replication sites suggesting its role in DNA synthesis (Xu et al., 1994). The cyclin E/cdk2 and cyclin A/cdk2 complexes are the active kinases in this phase. As the cell progresses from the

G1 to the S phase, the cyclin E/cdk2 complex is gradually replaced with the cyclin A/cdk2 complex. At the beginning of the S phase, the cyclin E/cdk2 complex initiates the assembly of the pre-replication complex, while the cyclin A/cdk2 complex regulates the initiation and progression of DNA synthesis, and ensures that only one round of DNA replication occurs in this phase (Coverley et al., 2002; Yam et al., 2002). During the mid-S phase of the cell cycle, the cyclin E1/cdk2 complex is inactivated by the p21, p27 or the p57 kinase. The cyclin A/cdk2 complex activates the FoxM1 family of transcription factors. The FoxM1 family of transcription factors promotes the expression of genes that are responsible for driving the the cells to enter mitosis (Chen et al., 2009). In the late S phase, cyclin A associates with cdk1 to form a cyclin A/cdk1 complex that remains stable until the late G2 phase of the cell cycle. The cyclin B1/cdk1 complex begins to appear and is confined to the cytoplasm in the late S phase of the cell cycle. DNA damage, if any, is detected and fixed in the S/G2 phase of the cell cycle. Double strand DNA breaks are recognized by Ataxia Telangiectasia Mutated (ATM) kinase. ATM phosphorylates and activates the checkpoint kinase2 (Chk2). The Chk2 arrests the cell cycle at the S phase, until all the damage has been repaired (Hirao et al., 2002).

G2 phase of the cell cycle. The cell prepares itself for mitosis in the G2 phase. During this phase, there is a rapid cellular growth that is marked by a very high rate of protein synthesis. There is a gradual accumulation of the cyclin B1/cdk1 complex through G2 phase of the cell cycle. At this point, the cyclin B1/cdk1 complex is restricted to the cytoplasm near the centrosome. It is kept in an inactive form by phosphorylation of cdk1 at Thr14 and Tyr15 by Wee-1 and Myt1 kinases (Mueller et al.,

1995; Parker, 1992). At the end of the G2 phase, CDC25 phosphatase dephosphorylates cdk1 at Thr14 and Tyr15, and activates the cyclin B1/cdk1 complex. Levels of the active cyclin B1/cdk1 complex reach a maximum just before the cell enters the M phase of the cell cycle.

M phase of the cell cycle. Polo-like kinases phosphorylate S133 and S147 of cyclin B1, facilitating its entry into the nucleus during the M phase of the cell cycle (Nigg, 2001; Toyoshima-Morimoto et al., 2001). Cyclin A is ubiquitinated and degraded in early metaphase. The mitotic phase is the shortest phase as well as the most fragile phase of the cell cycle. The cells in this phase are highly susceptible to death when exposed to various insults (Chan et al., 2012). The cyclin B1/cdk1 complex first appears on the centrosomes early in prophase and remains dominant until the end of metaphase (Jackman et al., 2003; Nurse, 1990). The cyclin B1/cdk1 complex is activated by CDC25B, and along with Polo-like kinase, it phosphorylates the nuclear lamins and an inner nuclear membrane. This results in chromosome condensation, breakdown of the nuclear membrane, centrosome separation, mitotic spindle assembly, Golgi fragmentation and mitochondrial fission during cell division (Li et al., 1997). Sister chromatid separation requires a cysteine proteinase, separase. Separase is kept in an inactivated state by a protein, securin, until all the chromatids in metaphase are in a proper orientation. The spindle assembly checkpoint (SAC) ensures that the sister chromatid separation is initiated in metaphase only after all the chromatids have established a correct bipolar attachment (Guttinger et al., 2009). SAC inhibits activation of anaphase promoting complex (APC/C) until all the errors in metaphase are fixed. At the end of

metaphase, both, the cyclin B1/cdk1 complex and securin, are ubiquitinated by APC/C and rapidly degraded by the proteasome degradation pathway (Guttinger et al., 2009).

During anaphase, the chromatin masses are compacted by a DNA binding protein, chromokinesin. The nuclear pore complex assembly is initiated during late anaphase, resulting in the formation of chromatin bound prepores. The endoplasmic reticulum membrane tubules also start binding to the chromatin surface. During telophase, the chromatids separate and the nuclear membrane is formed (Guttinger et al., 2009). Cytokinesis marks the end of cell cycle, where the cytoplasm of the parental cell is divided into two, each bound by its own cell membrane.

Role of cyclins C, H, K, and T in transcription. RNA polymerase II (RNA Pol II) forms a part of the pre-initiation complex (PIC) responsible for gene transcription. The carboxy-terminal domain (CTD) of RNA Pol II contains multiple heptapeptide repeats that can be targeted by the Cyclin/cdk complexes (Cisek & Cordon, 1989). Progressive changes in the phosphorylation status of the CTD play a crucial role in the timing of RNA Pol II activity, and sequential recruitment of various co-regulators (Lim & Kaldis, 2013).

The cyclin C/cdk8 complex is a component of the mediator complex that induces phosphorylation of the CTD domain of a large sub-unit of RNA Pol II (Knuesel et al., 2009). Cyclin C has been shown to cooperate functionally with c-myc and induce cell proliferation in a cytokine-independent fashion, and in the formation of the cell clusters in BAF-B03 cells (Liu et al., 1998). Furthermore, cyclin C was primarily shown to be

responsible for the induction of *cdc2* gene expression, thereby implicating its role in regulation of G1/S and G2/M phases of the cell cycle (Liu et al., 1998).

Cyclin H interacts with *cdk7* and the ring finger protein MAT1, and forms a *cdk*-activating kinase (CAK). CAKs are part of the general transcription factor II H (TFIIH), and phosphorylate the CTD of a large subunit of RNA Pol II. This phosphorylation of CTD by TFIIH results in promoter clearance and in progression of transcription from the pre-initiation to the initiation stage (Lolli & Johnson, 2005). The CAKs are also the regulators/activators of *cdk1*, *cdk2*, *cdk4* and *cdk6*.

Cyclin K can bind to both *cdk12* and *cdk9*. The cyclin K/*cdk12* complex regulates the phosphorylation of ser2 on the CTD domain of RNA Pol II, and is involved in the expression of long genes that have many exons (Blazek et al., 2011). The cyclin K/*cdk9* complex is a component of replication stress response, and appears on the chromatin in response to replication stress. Cyclin K/*cdk9* complex can interact with ATR kinases and other checkpoint signaling proteins, indicating its direct role in the checkpoint pathways that maintain the genome's integrity (Yu et al., 2010).

The cyclin T/*cdk9* complex forms a subunit of a positive elongation factor b (p-TEFB), and phosphorylates the CTD of a large subunit of RNA Pol II (Taube et al., 2002). This activation of RNA Pol II facilitates the transition from abortive to productive elongation of RNA transcripts during the process of transcription.

Deregulation of the cell cycle in cancer. Cancer is a multistage process where each stage involves different molecular, biochemical and cellular events, all of which contribute to malignant transformation. Substantial scientific evidence indicates that

cellular damage, e.g., radiation, oxidative stress, xenobiotics/chemicals, and viral infections, are some of the key factors associated with the development of cancer (Franco et al., 2008; Panayiotidis et al., 2008).

Unrestricted growth is one of the hallmarks of cancer and is associated with disordered cell cycle regulators. G1 is the only phase where cells can respond to extracellular mitogenic stimuli, and the advancement of the cell cycle depends on the balance of the proliferative and anti-proliferative signals. The cell cycle is controlled by the cyclin/cdk complexes, their inhibitors namely the INK4/cdk inactivating family of kinases (e.g., p15, p16, p8 and p19) and CKI family/cyclin/cdk inactivating kinases (e.g., p21, p27 and p57), CAKs (e.g., CDC25 phosphatase family), checkpoint proteins (e.g., CHK1 and CHK 2), and cdk-substrates (e.g., Rb proteins). A vast majority of cancers have abnormalities in one or more of these components, either due to an overexpression of positive regulators of cyclin/cdk functioning, or a decrease in negative regulators of cyclin/cdk functioning.

Extracellular cues that prompt the cell to divide are relayed through the receptors and ligands that reside in the cell membrane. Overexpression of these proteins due to mutations constitutively activates the signal transduction pathway. Resting cells and newly divided cells are triggered to undergo mitosis by the constant flow of mitogenic signals affecting downstream pathways such as the Notch signaling pathway, the NF- κ B signaling pathway and, the PI(3)K/AKT/mTOR signaling pathway.

In the Notch signaling pathway, the Notch intracellular domain targets cyclin D1, p21, c-myc and NF- κ B, and promotes cell proliferation. A deregulation of this signaling

is observed in breast cancer (O'Toole et al., 2013), T-cell leukemias (Guo et al., 2009), pancreatic and lung carcinomas (Abel et al., 2014; Hassan et al., 2013).

In the NF- κ B signaling pathway, NF- κ B regulates the expression of genes for manganese superoxide dismutase (SOD) (Xu et al., 2007). Deregulation of this pathway is seen in cancer where NF- κ B has been shown to induce cell proliferation by overexpressing cyclin D, and activating antiapoptotic genes e.g., Bcl-XL and Bcl2 (Malumbres & Barbacid, 2009).

The PI(3)K/AKT/mTOR signaling pathway has been shown to play an important role in cell proliferation and angiogenesis. Deregulation of this pathway is seen in most cancers, including colorectal cancer (Wang et al., 2013).

Deregulation of the proliferation pathway involving cyclin D/cdk4/6 and pRb is seen in a majority of cancers (Malumbres & Barbacid, 2009). Hyperactive cdk4 and cdk6 are seen in epithelial malignancies in endocrine tissues, mucosa, sarcomas, and leukemia. Mutation of cdk4 or cdk6 can inhibit either protein's binding to p16 in melanoma. Translocation in the promoter region of cdk6 gene leads to its overexpression in B-cell lymphocytic leukemia (Hayette et al., 2003; Malumbres & Barbacid, 2009). In all the above cancers, overexpression of cyclin D1/cdk4/6 coupled with inactivated INK and CIK resulted in hyperphosphorylation of pRB, releasing E2Fs that activate genes responsible for cell proliferation (Malumbres & Barbacid, 2009). Altered functions of p16, p15, and CIK have been seen in human breast cancers that were due to homozygous deletions or mutations in TP53. TP53 is a transcription factor for the CIK genes, and mutation of TP53 can inhibit its function to activate these genes (Musgrove et al., 1995).

In a recent study with breast cancer, almost 80% patients diagnosed with breast invasive carcinoma had copy number alterations (Zhang & Yang, 2014). In the majority of patients, loss of p16 activity was either due to homozygous deletions or heterozygous loss of one allele. The remaining patients demonstrated one or more alterations of the three genes previously discussed, *i.e.*, cyclin D, cdk, and INK/CIK. Cyclin D1 gene amplification was seen in 16% patients, and 2% of patients exhibited amplification of the cdk4 gene (Zhang & Yang, 2014).

The CDC25 family (CDC25A, CDC25B, and CDC25C) activates the cyclin E/cdk2, cyclin A/cdk2, and cyclin B1/cdk1 complexes through dephosphorylation of cdk1 and cdk2. These proteins play an important role in the G2/M transition by activating the cyclin B1/cdk1 complex (Boutros et al., 2011). Overexpression of CDC25 results in unscheduled activation of cyclin/cdk in primary breast cancer (Cangi et al., 2000; Ito et al., 2004), prostate cancer (Ngan et al., 2003) and non-small cell lung cancer (Sasaki, 2001).

A deregulated expression of cyclin E has been associated with a variety of cancers. An altered expression of cyclin E is seen in 18-22% of breast cancers (Stamatakis et al., 2010; Keyomarsi et al., 1994). In humans, six variants of cyclin E are found that bind to cdk2 and can carry out normal functions. A study by Porter and Keyomarsi determined that in breast tumors, a cyclin E1 splice variant is one-tenth of the total population of cyclin E1 mRNA (Porter & Keyomarsi, 2000). This splice variant displayed an altered substrate specificity and significantly changed its ability to bind to p21, demonstrating a constitutive expression of cyclin E in tumor cells (Porter & Keyomarsi, 2000). In ovarian

cancer patients, overexpression of cyclin E and cdk2 mRNAs was observed as a result of amplification of both cyclin E and cdk2 genes. In these patients, there was neither an aberrant expression of cyclin D1 and cdk4/6, nor any gain of chromosomes (Marone et al., 1998).

Cyclin B1 protein is highly overexpressed in 42% of breast carcinomas. In breast cancer tissue, this protein could be detected in early G1 phase of the cell cycle (Shen et al., 2004). High expression of cyclin B1 protein is associated with poor survival in breast cancer patients (Aaltonen et al., 2009). Breast cancers that test positive for nuclear cyclin B1 have been shown to be resistant to adjuvant therapy. Cyclin B1 is therefore used as a potent prognostic marker in human breast carcinomas (Suzuki et al., 2007). The cyclin B1/cdk1 complex is a key regulator of mitotic entry (Nigg, 2001), and can therefore be considered as a potential target for cancer therapeutics.

Transcriptional regulation is a crucial component of tumor progression, and metastasis (Ell & Kang, 2013). During cancer progression, dysregulation of oncogenic or tumor-suppressive transcription factors (TFs) have an effect on numerous steps of the metastasis cascade (Ell & Kang, 2013). Transcription factors such as c-myc, TP53, and Sp1 are some of the important cell cycle regulators.

C-myc is a ubiquitous TF and regulates the transcription of genes involved in the cell cycle progression and cell proliferation (Bretones et al., 2015). C-myc has been shown to be deregulated in most cancers (Vita & Henriksson, 2006). Triple-negative breast cancers show an elevated expression of c-myc and are associated with poor prognosis (Horiuchi et al., 2012).

Point mutations or missense mutation in checkpoint proteins like TP53 can lead to their inability to activate genes responsible for inducing apoptosis. Mutations in the TP53 gene lead to conformational changes in its protein. This results in abrogating its function as a TF for apoptosis-inducing gene(s), and instead has been shown to activate oncogenes (McDonald & el Deiry, 2000; Malumbres & Barbacid, 2009).

Sp1 (specificity protein 1) is a TF as well as a transcriptional activator that binds to GC-rich consensus sequences in the promoter region of a gene. It is necessary for expression and regulation of a variety of genes that are important in cell cycle regulation and development (Li & Davie, 2010). Sp1 is over-expressed in a number of cancers, including breast, gastric, pancreatic, lung and brain cancers, and is considered as a negative prognostic factor (Jiang et al., 2008; Guan et al., 2012; Yang et al., 2014; Guan et al., 2012; Beishline et al., 2012; Vizcaino et al., 2015).

Cancer chemoprevention. Cancer chemoprevention involves the utilization of natural, synthetic or biological substances to reverse, prevent or delay cancer development (Nair et al., 2007; Gopalakrishnan & Kong 2008; Steward & Brown, 2013; Jafari et al., 2014). DNA methylation, histone modification and miRNA expression play an important role in cancer development. Natural products and dietary constituents with chemopreventive potential have an impact on these processes (Gerhausar, 2013). Over the last few decades, extensive research has demonstrated that many natural phenolic compounds that exhibit health-beneficial effects have an impact on major cellular pathways and cell functions such as cell cycle regulation and DNA damage repair (Fraga et al., 2010; Gullet et al., 2010; Tan et al., 2011; Sung et al., 2012; Gerhauser, 2013).

Polyphenols and Cancer

Polyphenols are secondary metabolites in plants, synthesized from shikimate-derived phenyl propanoid and/or polyketide pathway(s), featuring multiple phenolic rings. Polyphenols are devoid of any nitrogen based functional groups in their most basic structure (Quideau et al., 2011). They are classified based on the number of phenolic rings and by the structural elements that bond these rings to one another. The main classes of polyphenols are the stilbenes, lignans, phenolic acids and flavonoids (Figure 3) (Manach et al., 2004).

Figure 3. Major classes of plant polyphenols.

The antioxidant ability of the polyphenols is due to the presence of the phenolic hydroxyl group that scavenges various reactive oxygen species, e.g., O[•]-, NO[•]- and, OH[•]-, and maintains healthy metabolic function. The presence of phenolic hydroxyl groups

enables the polyphenols to interact with proteins (Charlton et al., 2002; He et al., 2007), thereby affecting signal transduction pathways and cell proliferation (Meeran & Katiyar, 2008).

Role of polyphenols in cancer chemoprevention. Voluminous work on the polyphenols has demonstrated their effects on cancer cells. These compounds have been shown to affect a variety of signal transduction pathways including NF- κ B, AKT, MAPK, p53, androgen receptor, and estrogen receptor pathways (Lee et al., 2008). The polyphenols have been shown to inactivate cytosolic transcription factors, e.g., STAT3 and NF- κ B (Sun et al., 2012), and inhibit the JAK/STAT3/5 signaling pathway (Serra et al., 2014).

The inhibition of the JAK/STAT3/5 pathway results in phosphorylation of the tyrosine 705 in STAT3, thereby inactivating STAT3, which results in cell cycle arrest and apoptosis (Serra et al., 2014). Inactivation of STAT3 has been shown to negatively affect the activation of c-myc, cyclin D, anti-apoptotic proteins Bcl-XL, Bcl2, and survivin, in K562 myelogenous leukemia cells (Jung et al., 2013), and in several colorectal cancer cell lines, i.e., HCT 116, SWA480, and LOVO (Li et al., 2015).

The dietary polyphenols have also been shown to inhibit the AKT/MAPK/PKC and/or EGFR pathways and to inhibit the release of NF- κ B from IK- κ B/ NF- κ B complex. This results in failure to activate genes involved in inflammation and cell proliferation, ultimately resulting in cell cycle arrest and apoptosis (Granado-Serrano et al., 2010b; Pan et al., 2012; Sarkar & Li, 2004; Shukla & Gupta, 2007; Lee et al., 2008; Sun, 2012; Masuda et al., 2003; Serra et al., 2014).

Role of walnuts in cancer and other diseases. An antioxidant is defined as a redox active compound that limits oxidative stress by reacting non-enzymatically with a reactive oxidant (Blomhoff 2005). Whole walnuts are an excellent source of unsaturated fatty acids (linoleic and α -linolenic acid), tocopherols and melatonin (Kornsteiner et al., 2006), rich in potassium, calcium and magnesium (Pan et al., 2013), and have their highest antioxidant content in the pellicles, ranging from 10.8-33.3 mmol antioxidants/100 g (Blomhoff et al., 2006). Walnut seeds are rich in a variety of polyphenolic compounds (Carvalho, 2010; Li et al., 2006; Blomhoff et al., 2006). Walnuts with pellicles contains 16.25 mg gallic acid equivalent per 100 g fresh weight of polyphenols, and represent one of the highest polyphenol contents among nuts (Kornsteiner et al., 2006). Consumption of walnuts has been linked to a decrease in hypercholesterolemia, arteriosclerosis and cardiovascular disease (Kelly Jr. & Sabate, 2006; Banel & Hu, 2009). Walnuts also have a low glycemic index and hence are beneficial for people with diabetes mellitus (Pan et al., 2013).

Recently, some research groups have identified the polyphenolic compounds found in walnuts. With the help of high-speed counter-current chromatography (HSCCC) and liquid chromatography coupled with electrospray ionisation-ion trap-time of flight mass spectroscopy (ESI-IT-TOF-MS), Grace et al. (2014) identified a number of phenolic compounds from walnut extracts. In addition to gallic acid, dicarboxylic glucoside, hydrojuglone glucosides, catechin, pyrocynidin, and megastigmane glucosides, they also identified a total of 11 ellagitannins in a walnut extract. Regueiro et al. (2014) performed liquid chromatography coupled with electrospray ionization linear trap quadrupole

orbitrap mass spectrometry (LC-LTQ-Orbitrap) and reported 120 compounds, including hydrolysable and condensed tannins, flavonoids, phenolic acids, and ellagitannins. They were the first to identify eight new ellagitannins namely stenophyllanin C, malabathrin A, heterophyllin E, pterocarinin B, eucalbanin A, cornusiin B, reginin A and alienanin B in walnut extracts.

Ellagitannins are hydrolysable tannins that belong to the phenolic acids group of polyphenols. They exhibit antioxidant, antimicrobial, anticancer and anti-inflammatory properties (Lipinska et al., 2014). Ellagitannins are esters composed of hexahydroxydiphenic acids (HHDP) and a monosaccharide. Depending on the number of HHDP moieties, they are classified as monomeric (tellimagrandin I, tellimagrandin II), oligomeric (stenophyllanin C) or C-glycosidic (vescalgin).

In mouse models of cancer, a number of studies have demonstrated the effects of a whole walnut diet on the prevention and treatment of cancer. Nude mice injected with MDA-MB-231 cells and fed on a walnut diet had an 80% decrease in the growth rate of tumors when compared to the control mice fed on a diet containing corn oil (Hardman & Ion, 2008). In a well characterized mouse model for breast cancer, the C(3)1 TAg transgenic mouse, females are known to develop mammary gland cancer at a predictable rate (Maroulakou et al., 1994). In these mice, a whole walnut meal consumed by both the mother and her offspring was shown to slow the development as well as reduce the multiplicity of mammary gland cancers when compared to mice fed on a corn oil diet (Hardman et al., 2011). Since both walnuts and corn contain omega-3 fatty acids, this study concluded that omega-3 fatty acids probably were not responsible for inhibition of

mammary gland cancer, but the effect may have been due some other walnut components (Hardman et al., 2011).

Studies in mice with LNCaP prostate cancer xenografts fed with walnut diets reported a reduction in growth and size of the tumors. The tumors were one fourth the average size of the prostate tumors in mice fed on a diet devoid of walnuts (Reiter et al., 2013). In transgenic adenocarcinoma of the mouse prostate model (TRAMP), a walnut diet improved the lipid profile by increasing the HDL levels, reduced the size of the tumors, decreased levels of insulin growth factor-1, resistin, and LDL, and negatively regulated genes involved in gluconeogenesis (Davis et al., 2012). Despite the fatty acid content of high fat and whole walnut diets being constant, an absence of neuroendocrine and phyllode tumors was observed in mice fed with a walnut diet alone. This suggests that the effects observed were due to the whole walnuts and not due to specific fatty acid content in the diet (Davis et al., 2012). In prostate cancers, there was a decrease in both CIDEA (a cell death inducing DFFA-like effector) and MSMB (a microsemino protein B) mRNAs, and an increase in COX-2 and IGF-1 mRNAs. Kim et al. (2014) used the same mouse model and studied the effects of a walnut diet on the expression of CIDEA and MSMB. They reported that in mice fed with whole walnuts, walnut oil, or walnut-like fatty acids, the whole walnut diet demonstrated a decrease in IGF-1, an increase in CIDEA and MSMB mRNAs, with a concomitant decrease in COX-2 and IGF-1 mRNAs. These findings suggest that these effects were more likely due to walnut components other than fatty acids.

Recently, microRNA (miR) analysis demonstrated an increase in miR297 in nude mice that were injected with HT-29 human colon cancer cells, and fed with whole walnut as a source of fat (Tsoukas et al., 2015). A reduction in tumor size and a decrease in an inflammation related fatty acid, arachidonic acid, were observed in these mice. In humans, expression of miR297 in colorectal cancer is negatively related with the expression of a multi-drug resistant gene MRP-2 (Xu et al., 2012). Therefore, the walnut components are possibly responsible for the changes in the miRNA profiles that likely affect the target gene transcripts involved in anti-inflammation, anti-proliferation and apoptosis (Tsoukas et al., 2015).

All the above studies in mouse models have shown that components other than the fatty acids may have been responsible for the reduced tumor size in prostate, breast, mammary glands, and pancreatic cancers. Numerous studies with ellagitannins have demonstrated a cell cycle arrest at the G0/G1 phase, inhibition of cell proliferation and an induction of apoptosis, predominantly by affecting NF- κ B-signaling (Afaq et al., 2005; Adams et al., 2006; Kuo et al., 2007; Larrosa et al., 2006; Lansky & Newman 2007). Pomegranates are rich in an ellagitannin called punicalagin. In a multistage mouse skin carcinogenesis model, CD-1, an acetone extract of pomegranate seed coat and juice when applied topically on skin before 12-O-tetradecanoylphorbol-13-acetate (TPA) application, has been shown to inhibit the phosphorylation of ERK1/2, MAPK14, and JNK1/2. This resulted in inhibition of release of NF- κ B from the IK- κ B/ NF- κ B complex, and hence lessened the number and size of tumors as compared to mice that were treated with TPA alone (Afaq et al., 2005).

In Caco-2 colon cancer cells, punicalagin and ellagic acid demonstrated a dose and time-dependent inhibition of Bcl-XL, release of cytochrome C, and an activation of caspase 9 and caspase 3 that led to apoptosis via the mitochondria-mediated intrinsic pathway (Larrosa et al., 2006). A decrease in the expression of cyclin A1 and cyclin B1 indicated that the cell cycle was arrested at S and G2/M phases, while no effect of punicalagin or ellagic acid was seen in normal colon cell line-CCL-112CoN (Larrosa et al., 2006). These studies demonstrated that crude extracts as well as pure compounds, ellagitannin and punicalagin from pomegranates inhibit the cell signaling pathways responsible for cell proliferation and induce cell death (Larrosa et al., 2006).

A few *in vitro* studies, where cancer cells have also been treated with a defatted extract of walnuts, have been recently published (Carvalho, 2010., Salimi et al., 2012., Le et al., 2014). The defatted extracts are generated with methanol, acetone, petroleum ether, or chloroform, and have been shown to consist primarily of polyphenols such as ellagitannins and flavonoids.

Studies with methanolic extracts from the seeds and leaves of walnut have demonstrated a concentration dependent anti-hemolytic and an anti-proliferative effect on human renal cell lines, A498 and 769P, as well as on the colon cancer cell line CaCo2 (Carvalho, 2010). Two compounds, a flavonoid and a naphthoquinone, isolated from the chloroform fraction of walnut leaves, were reported to induce apoptosis and necrosis, and inhibit cell proliferation in a colon cancer cell line (HT-29), a breast cancer cell line (MCF7), and an oral squamous cell carcinoma cell line (BHY). These compounds were

shown to have no effect on the cell viability in the normal mouse fibroblast cell line NIH3T3 (Salimi et al., 2012).

Tellimagrandin-1, a walnut ellagitannin, upregulated the expression of connexin transcripts and proteins, restored the gap junctional communication in HeLa cells and attenuated the tumor phenotype (Yi et al., 2006). Cell cycle analysis demonstrated an increase in cells in the S phase of the cell cycle. Tellimagrandin I also inhibited the butyric acid (BA) induced differentiation of myelogenous leukemia cell line, K562 (Yi et al., 2004). In these cells, the expression of erythroid genes γ -globin and porphobilinogen (PBGD), GATA-1 and NF-E2 was downregulated, while the expression of GATA-2 inhibiting differentiation of erythroid and megakaryocytes was upregulated. This was indicative of an anti-tumor activity induced by tellimagrandin in the myelogenous leukemia cell line, K562 (Yi et al., 2004). In liver hepatocellular carcinoma HepG2 cells, the ellagitannin BJA3121, structurally related to tellimagrandin I, induced a time and dose dependent inhibitory effect on cell proliferation and regulation on the expression of miRNAs that were involved in regulation of differentiation (Wen et al., 2009). All the above studies demonstrate that tellimagrandin I was able to induce pathways that may regulate epigenetic mechanisms.

Megastigmane glucoside is found in a methanolic extract of walnuts, as well as in Laurel bay leaves. In human melanoma cell lines (A375, WM15 and SK-Mel28), megastigmane glycoside-Lauroside B has been shown to induce apoptosis via inhibiting the degradation of I κ B- α and binding of NF- κ B to the promoters of anti-apoptotic genes XIAP and c-FLIP (Panza et al., 2011).

Prostate cancer cells over-express peptidyl-prolyl *cis/trans* isomerase, PinI. Juglone has been shown to exert a strong inhibitory effect on Pin I in LnCaP and DU145 cells by suppressing cell proliferation (Kanaoka et al., 2015). The enlargement of tumors was suppressed when these cells were injected in mice and treated with juglone. Similarly, in ovarian cancer cells, SKOV3, juglone downregulated the expression of Cyclin D1, induced cell cycle arrest at the G1/S phase, as well as decreased the membrane metalloproteinase 2, and induced apoptosis through a mitochondrial pathway (Fang et al., 2015). Juglone glycoside, a derivative synthesized by addition of glycosidic moieties to the hydroxyl group(s) of juglone, has been shown to induce an anti-tumorigenic effect in the human promyelocytic leukemia cells, HL-60 (Fedorov et al., 2011).

Recently, a time and dose dependent effect of a WE on triple negative breast cancer cells, MDA-MB-231, was reported from our lab (Le et al., 2014). Decreases in mitochondrial membrane potential, loss of cytochrome C as well as change in intracellular pH were observed. These observed effects suggested that cell death occurred due to an intracellular mechanism of apoptosis. Cell cycle arrest at the G2 phase with a concomitant decrease in cyclin B1 was also observed (Le et al., 2014). The goal of our research was to determine whether the decrease in cyclin B1 protein in WE-treated MDA-MB-231 cells was transcriptional or translational.

Methods

Materials

A methanolic-WE was prepared as described by Le et al. (2014), and dissolved in DMSO at 100 mg/mL. The pure walnut compounds, tellimagrandin I and casuarictin,

were obtained from Mary-Ann Lila's laboratory at North Carolina State University (Grace et al., 2014). Gallic acid and quercetin were purchased from Sigma and Fisher Scientific, respectively. All the pure compounds were dissolved in 100% DMSO to yield 50 mM stock concentrations.

The MDA-MB-231 (human breast adenocarcinoma) cell line was obtained from American type culture collection (ATCC). Dulbecco's modification of Eagle's medium (DMEM) trypsin/EDTA solution was purchased from Corning. Fetal bovine serum (FBS) was purchased from Sigma, and antibiotic/ antimycotic solution was purchased from HyClone. Primary antibody to glyceraldehyde 3-phosphate dehydrogenase, anti-GAPDH (sc-25778), and secondary antibodies, namely, mouse-IgG-HRP (sc-2748) and rabbit-IgG-HRP (sc-2749), were purchased from Santa Cruz Biotechnology. Rabbit monoclonal anti-Cyclin B1 (Y-106, ab32053) was purchased from AbCam. Cycloheximide was purchased from Fisher Scientific, and a stock solution of 100 mg/mL was prepared in 100% DMSO.

Pierce BCA protein assay kit, TRI reagent (Ambion), SuperScript III First-Strand Synthesis SuperMix (Invitrogen) and Power SyBR Green Master Mix (Applied Biosystems) were purchased from Thermo Fisher Scientific. QuantiFlour® RNA system and GoTaq Green PCR master mix were purchased from Promega. Quantitative RT-PCR primer pairs for detection of gene expression were either obtained from PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>) or were designed using Primer BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Table 1 shows the list of primers that were synthesized by and purchased from Eurofins MWG Operon (Huntsville, AL).

Table 1. List of primer sequences for genes analyzed by real-time quantitative PCR

Gene	GeneBank ID	Forward primer sequence (5'→3')	Size (base pairs)
		Reverse primer sequence (5'→3')	
GAPDH	NM_001289745	TGTTGCCATCAATGACCCCTT	202
		CTCCACGACGTACTCAGCG	
Cyclin A*	NM_003914	ACATGGATGAACTAGAGCAGGG	95
		GAGTGTGCCGGTGTCTACTT	
Cyclin B1	NM_031966	CAGCTGGTTGGTGTCACTGCC	170
		AGAGGCCGACCCAGACCAAA	
Cyclin C	NM_005190	CATCCTCTTCCTTTTCGCCGT	182
		AGTGGGAGCTCTGCCAAAAG	
Cyclin D1*	NM_053056	GCTGCGAAGTGGAAACCATC	135
		CCTCCTTCTGCACACATTGAA	
Cyclin E1*	NM_001238	GCCAGCCTTGGGACAATAATG	104
		CTTGACGTTGAGTTTGGGT	
Cyclin H*	NM_001239	TGTTCCGGTGTTAAGCCAGCA	106
		TCCTGGGGTGATATTCCATTACT	
Cyclin K*	NM_001099402	AAAGCCATGTTGGTACTGGGA	141
		TGTAGCCCCAAACGTGTGC	
Cyclin T	NM_001240	AGCACTGGTGGGAGTATGTTG	168
		CTGTTCCCTCGGTCATCTGCTT	
Sp1*	NM_001251825	TGGCAGCAGTACCAATGGC	126
		CCAGGTAGTCCTGTCAGAACTT	
Ki67	NM_0024177	CAGCACGTCGTGTCTCAAGAT	179
		TTTCCCTGAFCACACTGTC	
MXD4	NM_006454	AACAGGTCTTCACACAACGAG	195
		CTGCTCCTTGATGCTCAGTG	
p65	NM_021975	CTGCCGGGATGGCTTCTATG	176
		CGTAGTCCCCACGCTCTCT	
Notch1	NM_017617	GCTGGACTGGTGAGGACTG	178
		AGCCCTCGTTACAGGGGTT	

*Primers from PrimerBank

Cell Culture and Treatment

Cells were grown and maintained in DMEM with high glucose, L-glutamine and sodium pyruvate, supplemented with 10% FBS and 1% antibiotic/antimycotic solution in a 37°C incubator with 5% CO₂ in a humidified atmosphere. Approximately 1x10⁶ MDA-MB-231 cells were plated in a 6-well format culture plate and grown overnight prior to

the treatment. The cells were treated with either vehicle control DMSO, 0.1 mg/mL cycloheximide, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, or 0.0325 mg/mL WE. To determine the effects of various compounds on cyclin B1 expression, MDA-MB-231 cells were treated with tellimagrandin I, casuarictin, gallic acid, or quercetin at the final concentration of 50 μ M.

Quantitative Western Analysis for Measuring Half-life of Cyclin B1

Approximately 1.0×10^6 MDA-MB-231 cells per well in a 6-well culture plate were grown overnight. They were then treated with either DMSO, 0.5 mg/mL WE, 0.5 mg/mL WE with 0.1 mg/mL CHX, or 0.1mg/mL CHX alone. Cellular proteins were collected 0, 1, 2, 3, 4, 5, and 24 h after DMSO and WE treatment. At each time point the growth medium was aspirated and cells were washed with cold PBS (1X). These cells were lysed in 250 μ L-300 μ L lysis buffer (50 mM Tris-Cl pH 7.5, 300 mM NaCl, 0.5% sodium deoxycholate, 5 mM EDTA, 1% NP-40, 1% SDS) along with 1x Halt protease inhibitor cocktail (Thermo Scientific). Cell lysates were transferred to a -80°C freezer for 30 min, thawed on ice, and then sonicated with 2-4 quick bursts. Cell debris was removed by centrifuging cell lysates at 15,000 rpm for 10 min. Protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following manufacturer's recommended protocol for 96-well microplates. Cell lysates were stored at -80°C until they were ready to use for subsequent experiments.

SDS-PAGE and Western Blot Analysis of Cyclin B1

For SDS-PAGE, 1.5 mm thick slab gels were prepared in house. In brief, a 10 mL resolving gel (10% acrylamide w/v) was made by adding 5 mL MilliQ H₂O, 2.5 mL 4X

lower buffer (1.5 M Tris HCl pH 8.8, 0.4% SDS), 2.5 mL of 40% acrylamide, 60 μ L of 10% APS, and 15 μ L of TEMED. The 5 mL stacking gel (4.2% acrylamide w/v) was made by adding 3.225 mL of MilliQ H₂O, 1.25 mL of upper buffer (0.5 M Tris HCl pH 6.8, 0.4% SDS), 525 μ L of 40% acrylamide, 25 μ L of APS, and 6 μ L of TEMED). These slab gels were used to separate proteins for western blotting.

Total protein (10 μ g) was prepared in 1X Laemmli buffer containing 125 mM Tris HCl pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.004% bromophenol blue (Laemmli, 1970). The proteins were denatured by heating at 95⁰C for 5 min, immediately chilled on ice for 5 min and collected after a quick centrifugation step at maximum speed. These proteins were resolved on 10% SDS-PAGE and then electrophoretically transferred to PVDF membrane (Immobilon-P) using 1X transfer buffer (25 mM Tris and 192 mM Glycine) at 250 mA for 1 h. After protein transfer, the membranes were washed with TBS (20 mM Tris HCl pH 7.5, 500 mM NaCl) for 5 min at room temperature. Membranes were blocked with 5% milk in TBS-T (TBS with 0.5% Tween-20) for 1 h at room temperature. The blots were probed with primary antibodies rabbit anti-GAPDH (detects a 38 kDa GAPDH protein) at 1:1000 dilution and mouse anti-Cyclin B1 (detects a 58 kDa Cyclin B1 protein) at 1:5000 dilution in 5% milk in TBS-T and incubated overnight on a rotary shaker at 4⁰C.

The membranes were washed with TBS-T (3 washes, 15 min per wash) and probed with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody and goat anti-mouse HRP (Santa Cruz Biotechnology) at 1:5000 dilution for 1 h at room temperature. Membranes were developed using HyGlo QuickSpray (Denville Scientific)

according to the manufacturer's protocol. Proteins were detected by chemiluminescence using the ImageQuant Las4000 imager system (GE Healthcare). An image processing program ImageJ (National Institutes of Health) was utilized to quantitate the relative densities of GAPDH and Cyclin B1 bands. ImageJ measures the values of threshold areas of Cyclin B1 to its GAPDH. A ratio of cyclin B1/GAPDH determines the final protein turnover rate at each time point when compared with their corresponding ratios at $t=0$.

Time Course Analysis of a WE Treatment on Expression of Cyclin B1, Other Cyclins, and Various Genes

Approximately 1.0×10^6 MDA-MB-231 cells per well were grown overnight in a 6-well culture plate and then treated with either DMSO or WE at the final concentration of 0.5 mg/mL for 0, 1, 2, 3, 4, 5, 6, 9, 12, and 24 h. At each time point, the cells were homogenized in 500 μ L TRI reagent solution. They were kept frozen at -80°C until they were ready for RNA isolation.

Dose Dependent Effects of a WE Treatment on the Expression of Cyclin B1

Approximately 1.0×10^6 of MDA-MB-231 cells per well were grown overnight in a 6-well culture plate, and then treated with DMSO or with the WE at 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, and 0.0312 5mg/mL for 24 h. The cells were homogenized in 500 μ L TRI reagent solution and kept frozen at -80°C until they were ready for RNA isolation.

Expression of Cyclin B1 Transcripts in Response to Pure Compounds

Approximately 1.0×10^6 MDA-MB-231 cells per well were grown overnight in a 6-well culture plate and then treated with DMSO, tellimagrandin I, casuarictin, quercetin,

or gallic acid at a final concentration 50 μ M for 24 h. The cells were homogenized in 500 μ L TRI reagent, and kept frozen at -80⁰C until they were ready for RNA isolation.

Extraction of Total RNA, First-strand cDNA Synthesis, and RT-qPCR

Total RNA was extracted from MDA-MB-231 cell homogenates as described above. Briefly, the cell homogenates were thawed at room temperature for 5 min. To this homogenate chloroform (200 μ L) was added and thoroughly mixed for 2 min. and incubated at room temperature for 3min. This mix was centrifuged at 12,000 x g for 15 min. at 4⁰C. The supernatant was collected in a fresh tube. A second extraction with chloroform (250 μ L) was carried out to ensure all traces of phenol and guanidine isothiocyanate were removed from the cell homogenate. RNA from the supernatant was precipitated with 250 μ L of 100% isopropanol and incubated at room temperature for 15 min. The samples were centrifuged for 15 min, at 12,000 x g at 4⁰C. The precipitate was washed by vortexing briefly with 500 μ L of 75% chilled ethanol and centrifuged at 12,000 x g for 15 min at 4⁰C. After decanting the ethanol, the pellet was air dried at room temperature for 15 min. The RNA pellet was resuspended in 25 μ L DEPC-treated-RNase free water and dissolved completely by incubating it at 55⁰C for 10 min. Total RNA concentration was determined with a fluorescent RNA-binding dye, the QuantiFluor® RNA system (Promega). A standard curve with a regression analysis was generated using a range of dilutions of 1.2kB RNA Standard (Promega). Using this regression equation ($R^2 = 0.95-0.99$), the concentration of RNA from the experimental material was calculated.

RNA (1 µg total) was used to carry out the first-strand complementary DNA (cDNA) synthesis reaction with SuperScript[®] III First-Strand Synthesis SuperMix (Invitrogen). Except for the reactions that were scaled down to 15 µL reaction volume, all the subsequent steps of first-strand cDNA synthesis with oligo (dT)₂₀ were followed according to manufacturer's instructions. Resulting cDNA was diluted 1:100 in sterile water and 3 µL was used for subsequent PCR reactions. Before carrying out quantitative real-time PCR, cDNA synthesis was confirmed with end-point PCR reactions performed in 20 µL reaction volume with GoTaq Green (Promega) master mix and GAPDH primer pairs. The cycling parameters consisted of one cycle of 95⁰C for 2 min followed by 40 cycles, each cycle comprised of 95⁰C for 15 sec, 60⁰C for 30 sec, and 72⁰C for 35sec, respectively. The PCR reaction ended with one final 2 min extension at 72⁰C. The resulting PCR products were checked on 1.5% agarose gels, with a 100kbp ladder as a standard to determine the molecular weight of the PCR products.

Real-time quantitative PCRs with genes of interest were amplified in an ABI 7300 real time PCR detection system (Applied BioSystem) using *Power SYBR*[®] Green PCR master mix (2X). All the qPCR reactions were carried out in a MicroAmp[®] optical 96-well reaction plates, sealed with MicroAmp[®] optical adhesive seal. The plates were spun down at 2000 rpm for 3 min. before placing them in the ABI-7300 instrument.

Each reaction mixture consisted of 100 ng each of forward and reverse primers, and 3 µL cDNA (diluted 1:100 from the first strand cDNA synthesis reaction). The volume of this mix (cDNA and primer pairs) was brought to 10 µL with sterile water. The final reaction volume was made to 20 µL by an adding 10 µL of 2 X-Power SYBR Master

mix. The qPCR reactions for each gene and treatment were performed in triplicate. Thermal cycling in ABI 7300 machine was programmed for qPCR with cycling parameters that consisted of 3 stages; stage 1 at 95⁰C for 10 min, followed by stage 2 of 40 cycles, where each cycle consisted of 95⁰C for 10 sec, followed by 60⁰C for 40 sec. A final stage 3 consisted of a dissociation cycle with 95⁰C, 15 sec; 60⁰C, 20 sec; 95⁰C, 15 sec; and 60⁰C for 20 sec. RT-qPCR data for each gene was normalized to the cycle threshold (Δ Ct) value of an endogenous gene, GAPDH obtained from the same sample. The delta-delta Ct ($\Delta\Delta$ Ct) value that determines the fold-change was calculated according to the User's Guide from Applied BioSystems. To calculate the change in expression ($\Delta\Delta$ Ct) of gene of interest with respect to time in cells treated with DMSO and WE; a '0 h' untreated sample served as a calibrator. The list of primer sequences used for analysis for changes in gene expression has been listed in Table 1.

Statistical Analysis

The regression analysis for calculating the half-life of the cyclin B1 protein after (WE+CHX) and CHX treatments was conducted in Microsoft Excel and the data were tested for significance using two-tailed Student *t*- test. A P value <0.05 was regarded as statistically significant. Univariate ANOVA test was conducted to detect effects of WE on the expression of Cyclin B1 in the first five hours of treatment. For this statistical test, significance level was set at $\alpha=0.05$.

RT-qPCR data with various concentrations of WE and with pure walnut compounds were analyzed using ANOVA in IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY). The significance level was set at $\alpha=0.05$. Treatment was the

independent variable and expression of Cyclin B1 was the dependent variable. To determine which treatments had statistically significant results, post-hoc tests (Tukey's HSD) were performed when the ANOVA delivered a statistically significant effect of treatment.

RT- qPCR data with 2 treatments (DMSO and WE), each with 9 data points, were evaluated using ANOVA in SPSS. Two-way ANOVA was conducted to examine the effect of treatments (DMSO and WE) and time on the mRNA levels. Both treatment and time were considered as the independent variables, and expression of gene as a dependent variable. The significance level was set at $\alpha = 0.05$. Time was not treated as a repeated measure factor/variable because independent samples were tested at each time point rather than the same sample being repeatedly measured. For each statistically significant main effect of time, Tukey's HSD post-hoc tests were performed to determine which time points had statistically different results.

Results

Treatment of MDA-MB-231 Cells with a WE Does Not Affect the Stability of Cyclin B1 Protein

Previous studies in our laboratory have demonstrated a dose and time-dependent effect on the expression of cyclin B1 protein. The decreased level of cyclin B1 protein may be responsible for G2/M cell cycle arrest (Le et al., 2014). Dietary compounds have been shown to affect transcriptional and protein degradation pathways (Yamamoto, 2001; Chen, 2005). The decrease in cyclin B1 may be due to the activation of a protein degradation pathway or inhibition of transcription. In eukaryotes, CHX interferes with

the translocation step by blocking the translational elongation step in protein synthesis. CHX does not directly interfere with the ubiquitin-proteasome degradation pathway and hence can be used as a tool to determine the half-life or stability of proteins in response to drug treatment. The stability of the cyclin B1 protein in MDA-MB-231 cells treated with a WE was evaluated by performing a CHX-chase assay.

MDA-MB-231 cells were treated with DMSO, CHX (100 μ g/mL), and a WE (0.5 mg/mL) both alone and in combination with CHX (0.1 mg/mL). Cellular proteins were collected after 0, 1, 2, 3, 4, 5, and 24 h in DMSO and WE-treated cells and after 1, 3, 5, and 24 h in WE+CHX and CHX-treated cells. No significant difference in the expression of cyclin B1 protein was evident in the first 5 h in DMSO or WE-treated breast cancer cells (Figure 4).

Two-way ANOVA demonstrated no effect of time and treatments (DMSO and WE) on the expression of cyclin B1 in the first 5 hours of treatment ($F_{\text{treatment}}(1, 5.674) = 0.071$, $P = 0.793$). There was no statistically significant effect of time ($F_{\text{time}} = (4, 5.674)$, $P = 0.158$) or treatment on the expression of Cyclin B1. There were also no interactions displayed between time and treatment ($F_{\text{time*treatment}} = (4, 5.674)$, $P = 0.690$).

Figure 4. Effect of a WE treatment on the relative quantity of cyclin B1 protein. Quantitative western analysis was performed on MDA-MB-231 cells treated with DMSO and a WE. No significant difference in the relative quantity of cyclin B1 protein was evident in cells treated with a WE and DMSO during the first 5 hours of treatment. Error bars represent a standard error of the mean ($\pm$ SEM) from four independent experiments.

Quatitative Western analysis with ImageJ revealed a time-dependent decrease in cyclin B1 and GAPDH proteins in both (WE+CHX) and CHX-treated cells (Figures 5A, 5B), indicating that CHX has an effect on protein synthesis within 3h of treatment.

Based on the regression analysis for each treatment and time, the halflife of the cyclin B1 protein in WE+CHX-treated cells was 4.38 h or 262.95 min, and in CHX-treated cells was 3.51 hours or 210.46 min. (Figure 5C). A two-tailed student's t-test (assuming unequal variance) indicated that there was no significant difference in the half-life of cyclin B1 in WE+CHX and CHX- treated cells ($P=0.235$). This indicates that the decrease in the relative quantity of the cyclin B1 protein may not be due to its degradation.

Figure 5. Effect of aWE treatment on the stability of cyclin B1 protein. Quantitative western analysis was performed on MDA-MB-231 cells treated with DMSO, WE, WE+CHX, and CHX (A). A time-dependent decrease in expression is seen in CHX-treated cells in presence or absence of WE (B). Half-life of cyclin B1 in cells treated with WE+CHX and CHX was calculated with the linear regression equation obtained for each treatment and time. Error bars represent the standard error of mean ($\pm$ SEM) from four independent experiments.

A Dose Dependent Effect in the Relative Expression of Cyclin B1 in Response to a WE Treatment

Previous studies in our lab (Le et al., 2014) have demonstrated a dose-dependent effect on the expression of cyclin B1 protein. The goal of our study was to determine if this phenomenon is observed with the cyclin B1 transcripts as well. MDA-MB-231 cells were treated for 24 h with a WE at concentrations of 0.03125 mg/mL, 0.0625 mg/mL, 0.125 mg/mL, 0.250 mg/mL, and 0.500 mg/mL. DMSO treatment served as vehicle

control. RT-qPCR was performed as mentioned in the Material and Methods section. After normalizing the Cyclin B1 expression with GAPDH, a change in expression of cyclin B1 with respect to concentrations of a WE was calculated. $\Delta\Delta C_t$ from DMSO treated cells served as a calibrator. A dose-dependent effect on the expression of cyclin B1 was evident, with a 90% decrease in the cyclin B1 transcripts being observed at the highest dose (0.5 mg/mL) of WE (Figure 6).

A statistically significant effect of the concentrations of WE on the abundance of the cyclin B1 transcripts was obtained by analyzing the RT-qPCR data with ANOVA ($F_{\text{Concentration}}(5, 48) = 57.623, P < 0.001$). Tukey's test revealed a significant effect of the treatment on the amount of the cyclin B1 transcripts.

The effect on the abundance of cyclin B1 varied significantly in DMSO treated cells, as well as in those treated with various concentrations of WE ($P_{\text{DMSO vs WE } 0.03125} < 0.001$, $P_{\text{DMSO vs WE } 0.0625} < 0.001$, $P_{\text{DMSO vs WE } 0.125} < 0.001$, $P_{\text{DMSO vs WE } 0.250} < 0.001$, and $P_{\text{DMSO vs WE } 0.500} < 0.001$). WE concentrations ranging from 0.03125 mg/mL to 0.0625 mg/mL ($P_{\text{WE } 0.03125 \text{ to WE } 0.0625} = 0.991$), 0.125 mg/mL to 0.250 mg/mL ($P_{\text{WE } 0.125 \text{ to WE } 0.250} = 0.153$), and 0.250 mg/mL to 0.500 mg/mL ($P_{\text{WE } 0.25 \text{ to WE } 0.5} = 0.932$), did not show a significant effect on the cyclin B1 transcripts. Significant dose-dependent effect of WE concentration was clearly observed in cyclin B1 transcripts from 0.0625 mg/mL to 0.125 mg/mL ($P_{\text{WE } 0.0625 \text{ vs WE } 0.125} = 0.029$), 0.250 mg/mL ($P_{\text{WE } 0.0625 \text{ vs WE } 0.250} < 0.001$), 0.5 mg/mL ($P_{\text{WE } 0.0625 \text{ vs WE } 0.500} < 0.001$), and from 0.125 mg/mL to 0.5 mg/mL ($P_{\text{WE } 0.125 \text{ vs WE } 0.50} < 0.015$).

Figure 6. Relative expression of the cyclin B1 transcripts in responses to various concentrations of a WE. Complimentary DNAs were synthesized from total RNA isolated from the MDA-MB-231 cells that were treated with vehicle control-DMSO and various concentrations of a WE for 24 h. The effect of a WE was determined by performing RT-qPCR on the cDNAs. Relative expression of cyclin B1 was normalized to the endogenous control GAPDH. Error bars represent the standard error of the mean ($\pm$ SEM) from three independent experiments.

Effect of WE, Tellimagrandin I, Casuarictin, Gallic acid, and Quercetin Treatments on the Transcription of Cyclin B1

Cell viability assays with MDA-MB-231 cells treated with tellimagrandin I, casuarictin, and gallic acid have been shown to induce 80%, 20%, and 0% cell death, respectively (Le et. al., 2014). The aim of our study was to determine whether treatment with tellimagrandin I and casuarictin had an affect on the transcription of cyclin B1. RT-qPCR was performed on the cDNAs synthesized from the MDA-MB-231 cells that were treated with DMSO, tellimagrandin I, casuarictin, gallic acid, and quercetin for 24 h. After normalizing the cyclin B1 expression with GAPDH, the change in the expression ($\Delta\Delta$ Ct) of cyclin B1 was calculated in MDA-MB-231 cells treated with various compounds. To calculate the relative expression of cyclin B1, the $\Delta\Delta$ Ct from DMSO treated cells served as a calibrator. An approximately 60% decrease in the relative

amount of cyclin B1 transcripts was observed with tellimagrandin I, while a 40% decrease in the relative amount of cyclin B1 transcripts was evident with casuarictin. Neither gallic acid nor quercetin affected the transcription of cyclin B1 (Figure 7).

The RT-qPCR data was analyzed by ANOVA in SPSS where Tukey's HSD revealed no effect on the amount of cyclin B1 transcripts in cells treated with DMSO, gallic acid ($P_{\text{DMSO vs Gallic}} = 0.114$), or quercetin ($P_{\text{DMSO vs Quercetin}} = 0.114$). A statistically significant decrease in the cyclin B1 transcripts was observed in cells treated with WE ($P_{\text{DMSO vs WE}} < 0.001$), tellimagrandin I ($P_{\text{DMSO vs Tellimagrandin I}} < 0.001$), and casuarictin ($P_{\text{DMSO vs Casuarictin}} < 0.001$). Both, tellimagrandin I and casuarictin ($P_{\text{tellimagrandin I to casuarictin}} = 0.261$) had similar effects on the transcription of cyclin B1.

Time Course Analysis of the Effect of WE treatment on the Transcription of Some of the Genes Involved in Cell Proliferation

A WE treatment negatively affects the expression of cyclins B1, D1, E1, and A1.

Based on CHX-chase assay, it is apparent that a WE does not affect the half-life of the cyclin B1 protein. Hence, the decrease in the cyclin B1 protein may be occurring due to the inhibition of transcription of cyclin B1. A time course analysis was performed in order to profile cyclin B1 transcripts at 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 9 h, 12 h, and 24 h in MDA-MB-231 cells treated with vehicle control DMSO and WE (0.5 mg/mL).

Figure 7. Relative expression of cyclin B1 transcripts in response to WE, tellimagrandin I, casuarictin, gallic acid, and quercetin treatments. RT-qPCR was performed on the cDNAs isolated from the MDA-MB-231 cells treated with vehicle control-DMSO, WE (0.5 mg/mL) and pure compounds (50 μ M each) for 24 h. Expression of cyclin B1 was normalized to the endogenous control GAPDH. A statistically significant effect of treatment on the abundance of cyclin B1 ($F_{\text{treatment (5, 47)}} = 62.745$, $P < 0.001$) was observed. Error bars represent the standard error of the mean ($\pm$ SEM) from three independent experiments.

RT-qPCR data for cyclin B1 gene was normalized to the cycle threshold (Δ Ct) value of an endogenous gene, GAPDH, obtained from the same sample. The fold-change ($\Delta\Delta$ Ct) values were calculated according to the User's Guide from Applied BioSystems. A '0 h' untreated sample served as a calibrator for calculating the $\Delta\Delta$ Ct in the expression of cyclin B1 with respect to time in cells treated with DMSO or WE. The relative expression (RQ) or fold-change in the expression of cyclin B1 was calculated using the formula: Fold-change = $2^{-\Delta\Delta\text{Ct}}$.

We observed a constant level of cyclin B1 transcripts in DMSO treated cells with a noticeable increase at 12 h and 24 h (Figure 8). Two-way ANOVA was conducted to examine the effect of treatments (DMSO and WE) and time on the mRNA levels. There

was a statistically significant time*treatment interaction, $F_{\text{time}*\text{treatment}}(8, 549) = 4.717$, $P < 0.001$. There were significant main effects of treatment ($F_{\text{treatment}}(1, 549) = 1023.58$, $P < 0.001$) and time ($F_{\text{time}}(8, 549) = 7.546$, $P < 0.001$) as well.

Figure 8. Effect of a WE treatment on the transcription of cyclin B1. RT-qPCR analysis was performed on cDNAs from MDA-MB-231 cells treated with DMSO and a WE (0.5 mg/mL). Cyclin B1 expression was normalized to the endogenous control GAPDH. Error bars represent standard error of the mean ($\pm$ SEM) from three independent experiments. Double asterisk indicates statistically significant ($P \leq 0.0001$) change compared to vehicle control-DMSO.

Tukey's HSD on time showed that when compared to 1h, there was a statistically significant effect in mRNA levels at 6 h and 12 h ($P_{1h \text{ vs } 6h} = 0.020$ and $P_{1h \text{ vs } 12h} = 0.011$, respectively), from 2h and 3h to 6h ($P_{2h \text{ vs } 6h} = 0.019$ and $P_{3h \text{ vs } 6h} = 0.006$, respectively); from 2, 3, 4, 5, 6, and 9 h to 12 h ($P_{2h \text{ vs } 12h} = 0.006$, $P_{3h \text{ vs } 12h} = 0.018$, $P_{4h \text{ vs } 12h} < 0.001$, $P_{5h \text{ vs } 12h} = 0.001$, $P_{6h \text{ vs } 12h} < 0.001$, and $P_{9h \text{ vs } 12h} < 0.001$, respectively) and from 6 h to 24 h ($P_{6h \text{ vs } 24h} = 0.001$).

Like cyclin B1, the G1phase specific cyclin, cyclin D1, revealed a severe reduction in the transcripts after the first hour of treatment (Figure 9). cyclin D1 was maintained at low levels through 24 h WE treatment. Two-way ANOVA exhibited time*treatment interaction ($F_{\text{time*treatment}} (8, 193) = 8.854, P < 0.0001$) as well as an effect of treatment ($F_{\text{treatment}} (1, 193) = 274.891, P < 0.0001$). Time also had a statistically significant effect on the expression of Cyclin D1 ($F_{\text{time}} (8, 193) = 7.497, P < 0.0001$). Tukey's HSD test displayed a statistically significant effect on the abundance of Cyclin D1 transcript from 1 h and 2 h to 3 h ($P_{1\text{h to } 3\text{h}} = 0.016$ and $P_{2\text{h to } 3\text{h}} = 0.035$ respectively); from 1, 2, 4, and 5 h to 6 h ($P_{1\text{h vs } 6\text{h}} < 0.001$, $P_{2\text{h vs } 6\text{h}} < 0.001$, $P_{4\text{h vs } 6\text{h}} = 0.003$ and $P_{5\text{h vs } 6\text{h}} = 0.001$, respectively); 3 h and 6 h to 12 h ($P_{3\text{h vs } 12\text{h}} = 0.002$ and $P_{6\text{h to } 12\text{h}} = 0.001$ respectively); 6 h to 9 h ($P_{6\text{h vs } 9\text{h}} = 0.001$) and 12 h to 24 h with $P_{12\text{h to } 24\text{h}} < 0.001$.

Amplification with the S phase cyclin, cyclin E1, demonstrated a more than 50% reduction in transcription after the first hour of WE-treatment and remained constant through 24 h (Figure 10). The expression of cyclin E1 in DMSO treated cell also stayed constant through 24 h treatment in time*treatment interaction ($F_{\text{time*treatment}} (8, 142) = 0.213, P = 0.988$).

Figure 9. Effect of a WE treatment on the transcription of cyclin D1. RT-qPCR analysis was performed on cDNAs from MDA-MB-231 cells treated with DMSO and a WE (0.5 mg/mL). Expressions of cyclin D1 was normalized to the endogenous control GAPDH. The error bars represent standard error of the mean ($\pm$ SEM) from three independent experiments. Asterisks indicate statistically significant ($P \leq 0.0001$) change compared to vehicle control-DMSO.

A statistically significant effect of treatment and time demonstrated that a WE exerts its effect by affecting the number of detectable transcripts by qPCR ($F_{\text{treatment}} (1, 142) = 165.11, P < 0.0001$; $F_{\text{time}} (8, 142) = 2.866, P = 0.006$). Although two-way ANOVA demonstrated a significant effect of time, since Tukey's HSD did not detect which time points differed, LSD was used instead. The LSD resulted in statistically significant values displaying the effects of time on the expression of cyclin E1. A significant increase in the transcripts was noticeable from 1 h to 9, 12, and 24 h ($P_{1h \text{ vs } 9h} = 0.006, P_{1h \text{ vs } 12h} = 0.023, P_{1h \text{ vs } 24h} = 0.026$); from 2 h to 9, 12, and 24 h ($P_{2h \text{ vs } 9h} = 0.004, P_{2h \text{ vs } 12h} = 0.014, P_{2h \text{ vs } 24h} = 0.016$); from 3 h to 9, 12, and 24 h ($P_{3h \text{ vs } 9h} = 0.004, P_{3h \text{ vs } 12h} = 0.014, P_{3h \text{ vs } 24h} = 0.016$); from 4 h to 9, 12, and 24 h ($P_{4h \text{ vs } 9h} = 0.013, P_{4h \text{ vs } 12h} = 0.042, P_{4h \text{ vs } 24h} = 0.048$); from 5 h to 9, 12, and 24 h ($P_{5h \text{ vs } 9h} = 0.004, P_{5h \text{ vs } 12h} = 0.014, P_{5h \text{ vs } 24h} = 0.016$);

from 2 h to 9, 12, and 24 h ($P_{2h \text{ vs } 9h}=0.004$, $P_{2h \text{ vs } 12h}=0.014$, $P_{2h \text{ vs } 24h}=0.016$); and from 6 h to 9 h with $P_{6h \text{ vs } 9h}=0.0027$.

Figure 10. Effect of a WE treatment on the transcription of cyclin E1. RT-qPCR analysis was performed on cDNAs from MDA-MB-231 cells treated with DMSO and WE (0.5 mg/mL). Expression of cyclin E1 was normalized to the endogenous control GAPDH. The error bars represent standard error of the mean ($\pm$ SEM) from three independent experiments. Asterisks indicate statistically significant ($P \leq 0.0001$) change compared to vehicle control-DMSO.

Cyclin A1 is an S phase as well as a G2 phase cyclin. Like cyclin D1, cyclin E1 and cyclin B1, cyclin A1 is also affected by WE treatment. Nearly 70% decrease in the transcripts was evident after the first hour of WE treatment (Figure 11). We did not observe a statistically significant effect of time, as well as time*treatment interactions ($F_{\text{time}}(8, 193)=0.470$, $P=0.876$; and $F_{\text{time*treatment}}(8, 143)=1.418$, $P=0.157$). But effect of the treatment was statistically significant ($F_{\text{treatment}}(1, 143)=61.048$, $P<0.001$). Tukey's HSD test for simple main effects did not display any statistically significant effect of time within the treatments.

Figure 11. Effect of a WE treatment on the transcription of cyclin A1. RT-qPCR analysis was performed on cDNAs from MDA-MB-231 cells treated with DMSO and WE (0.5 mg/mL). Expressions of cyclin A1 was normalized to the endogenous control GAPDH. Error bars represent standard error of the mean ($\pm$ SEM) from three independent experiments. Asterisks indicate statistically significant ($P \leq 0.0001$) change compared to vehicle control, DMSO.

A WE treatment negatively affects the transcription of cyclins C, H, K, and T.

In addition to phase-specific cyclins, e.g., cyclin A1, cyclin D1, and cyclin E1, qPCR was carried out with cyclin C, cyclin H, cyclin K, and cyclin T. RT- qPCR was carried out with cDNAs prepared from MDA-MB-231 cells treated with DMSO and WE and collected after 1, 2, 3, 4, 5, 6, 9, 12, and 24 h, respectively. The change in expression ($\Delta\Delta Ct$) of cyclins with respect to time was calculated. A ‘0 h’ untreated sample served as a calibrator.

When compared to DMSO treated cells, WE-treated MDA-MB-231 cells showed more than an 85% reduction in both cyclin C and cyclin H transcripts after the first hour of treatment (Figures 12A and 12B). Two-way ANOVA tests demonstrated statistically insignificant time*treatment interaction in cyclin C ($F_{\text{time*treatment}}(8, 110) = 0.197$, $P=0.991$), and a significant effect of treatment ($F_{\text{treatment}}(1, 110) = 85.057$, $P \leq 0.0001$), but

not a significant effect of time ($F_{\text{time}} (8, 110) = 1.050, P=0.403$). Cyclin H displayed no significant time*treatment interaction with $F_{\text{time*treatment}} (8, 140) = 1.558, P=0.143$. An effect of both time and treatment on the levels of mRNA was observed with $F_{\text{treatment}} (1, 140) = 333.670, P\text{-value} \leq 0.0001$ and $F_{\text{time}} (8, 140) = 2.620, P=0.011$ respectively. Tukey's HSD revealed a significant difference in the abundance of cyclin H mRNA from time 6 h to 24 h ($P_{6\text{h vs } 24\text{h}}=0.032$) and from 12 h to 24 h ($P_{12\text{h vs } 24\text{h}}=0.006$).

Figure 12. Effect of a WE treatment on the transcription of cyclin C (A) and cyclin H (B). RT-qPCR analysis was performed on cDNAs from MDA-MB-231 cells treated with DMSO and WE (0.5 mg/mL). Expressions of cyclin C and cyclin H were normalized to the endogenous control GAPDH. The error bars represent standard error of the mean ($\pm$ SEM) from three independent experiments. Asterisks indicate statistically significant ($P<0.001$) change compared to vehicle control-DMSO.

Similar to the effect of a WE-treatment on expression of cyclin C and cyclin H, we observed effects of a WE-treatment on the expression of cyclin K and cyclin T transcripts (Figures 13A and 13B). A decrease in mRNA levels of these cyclins was observed after 1 h of a WE treatment. RT-qPCR data for cyclin K analyzed with two-way ANOVA displayed a statistically significant time*treatment interaction with $F_{\text{time*interaction}} (8, 144) = 3.20, P=0.002$. Significant effects of time ($F_{\text{time}} (8, 144) = 2.506, P=0.014$) and treatment ($F_{\text{treatment}} (1, 144) = 323.74, P<0.001$) were also observed. Tukey's HSD test

demonstrated an increase in abundance of cyclin K transcripts from 1 h treatment to 4 h ($P_{1h \text{ vs } 4h}=0.022$), 5 h ($P_{1h \text{ to } 5h}=0.021$), and 12 h ($P_{1h \text{ vs } 4h}=0.005$).

Figure 13. Effect of a WE treatment on the transcription of cyclin K (A) and cyclin T (B). RT-qPCR analysis was performed on cDNAs from MDA-MB-231 cells treated with DMSO and a WE (0.5mg/mL). Expressions of cyclin K and cyclin T were normalized to the endogenous control GAPDH. The error bars represent standard error of the mean ($\pm$ SEM) from three independent experiments. Asterisks indicate statistically significant ($P<0.001$) change compared to vehicle control-DMSO (P -value <0.001)

Two-way ANOVA analysis with cyclin T demonstrated a statistically significant time*treatment interaction with $F_{\text{time*treatment}}(8, 138)=6.945$, $P<0.001$. Like cyclin K, a statistically significant effects of both time ($F_{\text{time}}(8, 138)=3.315$, $P=0.002$) and treatment ($F_{\text{treatment}}(1, 138)=247.601$, $P<0.001$) are also observed in cyclin T.

Multiple comparisons with Tukey's HSD presented a statistically significant change in mRNA levels from 1h to 3, 4, and 5 h ($P_{1h \text{ vs } 3h}=0.025$, $P_{1h \text{ vs } 4h}=0.035$ and $P_{1h \text{ vs } 5h}=0.027$, respectively); and from 3 h to 24 h with $P_{3h \text{ vs } 24h}=0.047$.

Relative expressions of Notch1, RelA/p65, and Ki-67 in response to a WE treatment. To determine if a WE affects the transcription of the genes other than the cyclins, we selected three genes, i.e., Notch1, RelA/p65, and Ki67. Notch1 is reported to be routinely deregulated in many cancers including breast cancer. RelA/p65, a subunit of

NF- κ B, is a ubiquitous transcription factor, and is known to be downregulated by many dietary compounds. Ki67 is a cell proliferation marker that is observed in all dividing cells (Pan et al., 2012; Granado-Serrano et al., 2010a; Fantini et al., 2015). RT-qPCR was performed on the cDNAs from MDA-MB-231 cells treated with DMSO and WE. The cDNAs were collected at all the nine time-points and the fold-change was calculated as described above.

After the initial 50% decrease in transcripts in the first hour of treatment, Notch1 expression was seen to recover within 5 h to levels similar to Notch1 expression in DMSO-treated cells (Figure 14). RelA/p53 expression was maintained at almost similar levels in both DMSO as well as WE-treated MDA-MB-231 cells (Figure 14B). RT-qPCR results were analyzed with two-way ANOVA to examine the effect of treatments (DMSO and WE) and time on the mRNA levels and to determine if there was an interaction between time and the treatment.

Neither a statistically significant time*treatment interaction, $F_{\text{time*treatment}} (8, 143) = 1.435, P=0.187$, nor any effect of treatment, $F_{\text{treatment}} (1, 143) = 1.518, P=0.220$ was seen with Notch1. There was a significant main effect of time with $F_{\text{time}} (8, 143) = 4.769, P<0.001$. According to Tukey's HSD, results differed between 1 h and 5 h and between 2 h and 5 h ($P_{1h \text{ vs } 5h} = 0.011$ and $P_{2h \text{ vs } 5h} = 0.02$, respectively), between 5 h and 9 h and between 5 h and 12 h ($P_{5h \text{ vs } 9h} = 0.043$ and $P_{5h \text{ vs } 12h} < 0.001$, respectively), and between 6 h and 24 h ($P_{6h \text{ vs } 24h} = 0.002$).

Figure 14. Effect of a WE treatment on the transcription of Notch1 (A) and RelA/p65 (B). RT-qPCR analysis was performed on cDNAs from MDA-MB-231 cells treated with DMSO and WE (0.5 mg/mL). Gene expression was normalized to the endogenous control GAPDH. The error bars represent standard error of the mean ($\pm$ SEM) from three independent experiments.

With RelA/p65, there was no significant time*treatment interaction with $F_{\text{time*treatment}}$ (8, 144)=1.24, $P=0.279$, but there was a significant effect of treatment with $F_{\text{treatment}}$ (1, 144)=2.886, $P=0.002$. Similar to Notch1, a statistically significant effect of time on the expression of RelA/p65 mRNAs was seen ($F_{\text{time}}=8, 144)=3.838$, $P<0.0001$). Multiple comparisons with Tukey's HSD revealed a statistically significant effect of the time from 4, 5, and 6 h to 24 h with $P_{4\text{h vs }24\text{h}}=0.025$, $P_{5\text{h vs }24\text{h}}=0.001$, and $P_{6\text{h vs }24\text{h}}=0.004$, respectively.

After the first hour of a WE treatment, a 90% decrease in Ki67 transcripts was detected in MDA-MB 231 cells. This decrease in Ki67 transcripts remained the same through 24 h of a WE-treatment (Figure 15), indicating a drastic effect on the process of cell division. Two-way ANOVA revealed a statistically significant time*treatment interaction with $F_{\text{time*treatment}}$ (8, 137)=3.067, $P=0.003$, as well as a significant main effect of treatment ($F_{\text{treatment}}$ (1, 137)=937.673, $P<0.001$). We observed a constant expression of Ki67 through 24 h in cells treated with DMSO or WE (F_{time} (8, 137)

=1.035, $P=0.413$), indicating that there was no effect of time on the mRNA expression within each treatment.

Figure 15. Effect of a WE treatment on the transcription of cell proliferation marker Ki67. RT-qPCR analysis was performed on cDNAs from MDA-MB-231 cells treated with DMSO and WE (0.5 mg/mL). Ki-67 expression was normalized to the endogenous control GAPDH. The error bars represent standard error of the mean ($\pm$ SEM) from three independent experiments. (P -values ≤ 0.05). Asterisks indicate statistically significant ($P < 0.0001$) change compared to vehicle control-DMSO.

Relative expression of the transcription factors, Sp1, and MXD4 in response to a WE treatment. Abundance of the transcripts of two transcription factors, Specificity protein 1 - Sp1 and Max-Interacting Transcriptional Repressor-4 - MXD4, were investigated. Our RT-qPCR analysis demonstrates that WE exerts its effect by repressing Cyclin B1 transcription. The goal of this experiment was to discern if the decrease of Cyclin B1 transcripts is associated with a change in expression of SP1 and/or MXD4.

Real-time qPCR was carried out with the cDNAs prepared from MDA-MB-231 cells treated with DMSO and a WE and collected after 1, 2, 3, 4, 5, 6, 9, and 12 h (as described

in Materials and Methods). When compared to DMSO, expression of Sp1 mRNA decreased to ~50% after first hour and ~ 40% after 24 h (Figure 16A) of a WE-treatment.

Figure 16. Effect of a WE treatment on the transcription of Sp1 (A) and MXD4 (B). RT-qPCR analysis was performed on cDNAs from MDA-MB-231 cells treated with DMSO and WE (0.5 mg/mL). Expressions of Sp1 and MXD4 were normalized to the endogenous control GAPDH. The error bars represent standard error of the mean ($\pm$ SEM) from three independent experiments (P -value ≤ 0.05).

Two-way ANOVA was conducted on the RT-qPCR results to examine the effect of treatments (DMSO and WE) and time on Sp1 mRNA levels. A statistically significant effect of treatment was evident with $F_{\text{treatment}} (1, 142) = 101.876, P < 0.0001$. No significant time*treatment interaction was noticed with $F_{\text{time*treatment}} (8, 142) = 0.366, P = 0.937$. No significant effect of time was evident since a constant pattern of expression of Sp1 mRNA was observed through out the time course in cells treated with DMSO and WE, respectively ($F_{\text{time}} (8, 142) = 1.637, p = 0.119$).

Expression of MXD4, a MAX-interacting transcriptional repressor, was analyzed by RT-qPCR. The MDA-MB-231 cells treated with both DMSO and WE exhibited no effect on the expression of MXD4 transcripts (Figure 16B). Two-way ANOVA did not demonstrate any statistically significant time*treatment interaction ($F_{\text{time*treatment}} (8, 144)$

=0.300, P=0.965). Neither the effect of treatment ($F_{\text{treatment}} (1, 144) = 3.709$, P=0.056) nor that of time ($F_{\text{time}} (8, 144) = 1.758$, P=0.09) were statistically significant.

Discussion

WE-treatment in MDA-MB-231 cells has been shown to induce cell death via apoptosis and a decrease in the levels of the cyclin B1 protein (Le et al., 2014). We did not determine the molecular mechanisms induced by WE-treatment. The findings in this study have provided the supporting evidence that WE affected not only the transcription of cyclin B1, but also affected the transcription of cyclins D1, A, E1, C, H, K, and T. Furthermore, there was a decrease in the transcription of cell proliferation marker Ki67 and transcription factor Sp1. We did not observe any significant effect on the transcription of Notch1, RelA/p65 and MXD4.

A WE Treatment Does Not Affect the Half-life of Cyclin B1 Protein

In tumors, inhibition of proteasome activity has been shown to induce apoptosis (Adams, 2003). In Jurkat T leukemia cells, dietary polyphenols have been shown to accumulate high molecular weight ubiquitylated Bax and I κ B- α as early as 1h post-flavonoid treatments (Chen et al., 2004; Chen et al, 2005). This accumulation of Bax and I κ B- α in flavonoid-treated cells indicated an inhibition in the proteosomal degradation pathway that resulted in an induction of apoptosis. Our western analysis did not show a high molecular weight band with cyclin B1 antibodies, indicating that the cyclin B1 protein may not be degraded via the proteosomal pathway. Studies with cell-free translation of the cyclins, cyclin A and cyclin B1 in *Xenopus* egg extracts and their

interactions with cdk1 indicated that cyclin B1 forms a very stable complex with cdk1 that has a half-life of 15h (Kobayashi et al., 1994). We detected no effect on the amount of Cyclin B1 protein in the first few hours in MDA-MB 231 cells treated with WE. Given the fact that cyclin B1/cdk1 complexes are stable for more than 5h, it is quite possible that this complex was stable in presence of WE as well. In this study, we saw a decrease in the levels of cyclin B1 protein after 24h post WE-treatment indicating that the decrease is perhaps due to an insufficient transcription of cyclin B1.

Treatments with WE, Tellimagrandin I, and Casuarictin Affect the Transcription of Cyclin B1 in MDA-MB-231 cells

Cyclin B1/cdk1 complex is an important kinase in the mitotic phase of the cell cycle. This complex directs the cells to undergo mitosis by phosphorylating proteins involved in chromosome condensation, nuclear envelope breakdown, spindle assembly, centrosome separation, Golgi fragmentation, and mitochondrial fission (Nurse, 1990). Any decrease in the cyclin B1 transcripts results in a corresponding decrease in the production of the cyclin B1 proteins. Decreases in cyclin B1 proteins impede its function to direct the cells into mitosis and results in the cell cycle arrest at G1/S phase. A prolonged G1 and S phase arrest may either induce senescence or genomic instability, ultimately leading to cell death (Yi et al, 2006). It is plausible that such phenomenon occurs in WE-treated MDA-MB-231 cells, where we see almost a 90% decrease in the relative amount of cyclin B1 transcripts.

Tellimagrandin I has been shown to induce connexin 43 and enhance the gap junctional communication, as well as attenuate the cell growth in HeLa cells (Yi et al,

2006). Additionally, the cell cycle was arrested at the G0/G1 and the G2/M phases. This arrest in the cell cycle was shown to be accompanied with an increase in the duration of the S phase and a decrease in cyclin A (Yi et al, 2006).

Previous studies from our lab have demonstrated effects of two ellagitannins, tellimagrandin I and casuarictin, isolated from a WE on the viability of MDA-MB-231 breast cancer cells. These studies demonstrated that while tellimagrandin I was able to induce ~85% cell death, only a fraction of cells died when treated with casuarictin. Compared to tellimagrandin I, no significant cell death was induced when MDA-MB-231 cells were treated with casuarictin. In this study, in MDA-MB-231 cells treated with either both tellimagrandin I or casuarictin, we observed a significant decrease in the transcription of cyclin B1.

Taken together, these studies indicate that despite a decrease in the transcription of cyclin B1 with compounds, tellimagrandin I and casuarictin; their effects on the viability of MDA-MB-231 cells were different. Tellimagrandin I treatment decreased cyclin B1 transcripts and induced cell death, while casuarictin treated cells decreased number of cyclin B1 transcripts but showed no effect on cell viability. It is possible that in MDA-MB-231 cells, pure compound treatments, either tellimagrandin I or casuarictin each induced a separate molecular pathway. To our knowledge our lab is the first lab to study the effects of tellimagrandin I and casuarictin on the transcription of cyclin B1 in MDA-MB-231 cells.

A WE Treatment Negatively affects Transcription of Ki67, Cyclins B1, D, E1, A, C, H, K, and T

Expression of Ki67 is strictly associated with the cell proliferation. It is routinely used as a prognostic marker in early breast cancer (de Azambuja, 2007), non-small cell lung cancer, acute lymphoblastic leukemia, and cervical cancer (Bruey et al., 2009, Pan et al., 2015). Ki67 is also used as a treatment monitoring marker in various cancers (Gerdes, 1990; Jonat & Arnold 2011; Tsumagari, 2015). It is absent in the resting cells, and is found in all active phases of the cell cycle i.e., G1, S, G2, and M phase, (Scholzen & Gerdes, 2000; Endl & Gerdes 2000; Kanavaros et al., 2001). Ki67 protein has been shown to bind to heterochromatin protein 1 (HPI) with high affinity and co-localize in the interphase nuclei. This suggests that it has a role in regulating high-order chromatin structure, plausibly by maintaining the heterochromatin domains in the interphase nuclei (Scholzen et al., 2002). In proliferating cells, Ki67 protein is seen associated with ribosomal transcription machinery in the interphase nuclei (Scholzen et al., 2002). It is also present in nucleolar organizing region (NOR) in the mitotic phase of cell cycle and is associated with the DNA polymerase I transcription machinery, suggesting its role in the transcription of rDNA. Low levels of Ki67 protein is seen associated with the rDNA in quiescent cells (Bullwinkel et al., 2006). A dose-dependent effect of small-inhibitory RNA (siRNA) targeted against Ki67 with a concomitant induction of apoptosis has been reported in human renal carcinoma cell line 760-0 (Zheng et al., 2006). We were interested in determining whether WE-treatment was able to exert its effect on the transcription of Ki67 in MDA-MB-231 cells.

Nearly 80% repression in the transcription of Ki67 was seen 1 h after treating MDA-MB-231 cells with a WE. These cells showed a decrease in cyclin B1, D, E, and A. Furthermore, they also showed a profound decrease in the expression of cyclins C, H, K, and T. These cyclins are involved in activation of RNA Pol II. Each of these cyclins has a very important role in regulation of RNA Pol II-based transcription. There are multiple repeats of the heptamer sequences on the CTD of the large subunit of the RNA Pol II. Throughout the transcription, phosphorylation activities executed by these cyclin/cdk complexes are required to achieve the dynamic patterns of phosphorylation on the CTD. The phosphorylation of proteins by cyclin /cdk complexes are restricted during each phase of the transcription cycle that drive a stepwise progression from pre-initiation, initiation, elongation, and termination (Buratowski, 2009; Lim & Kaldis, 2013). It is plausible that there is a decrease in transcription of cyclins C, H, K and T which affects the activation of RNA Pol II in cells treated with a WE, resulting in an overall reduction in the transcription of genes important in cell division.

It has been demonstrated that in response to external stimuli, there is an intricate convergence of both transcriptional and mRNA turnover events that may directly influence the steady-state mRNA levels (Cheadle et al., 2005). We believe that the acute effect on the Ki67 and cyclin transcripts observed in WE-treated MDA-MB-231 cells is either due to a post-transcriptional rapid turnover of poly(A) mRNA in response to cellular stress caused by WE treatment, or due to a rapid inhibition of newly expressed RNA.

A WE Treatment has No Effect on the Transcription of Notch1 and RelA/p65

Notch1 targets NF- κ B, cyclin D1, Akt, cyclooxygenase-2, extracellular signal-regulated kinase, matrix metalloproteinase-9, and c-myc. Notch1 also plays an important role in cell proliferation, differentiation, and apoptosis (Oswald et al., 1998; Ronchini et al., 2000; Weng et al., 2006; Guo et al., 2011). Notch1 is constitutively activated in lymphoblastic leukemia cell line DND-41. Quercetin has been shown to suppress the expression and activity of Notch 1 in these cells (Kawahara et al., 2009). A dose-dependent effect of genistein on the expression of Notch1, RelA/p65, cyclin B1, and anti-apoptotic proteins Bcl-2 and Bcl-XL was seen in MDA-MB-231 cells (Pan et al., 2012). A corresponding decrease in NF- κ B was also observed in MDA-MB-231 cells that were treated with Notch1 siRNA, demonstrating that Notch1 signaling is involved in the activation of NF- κ B (Pan et al., 2012).

RelA/p65, a subunit of NF- κ B, is a ubiquitous transcription factor that is involved in several biological processes. RelA/p65 plays an important role in inflammation, cell proliferation, and differentiation and can induce or inhibit apoptosis (Oswald et al., 1998; Bradford et al., 2014; Vermeulen et al., 2002). NF- κ B is a heterodimeric or homodimeric complex formed from five subunits (RelA/p65, Rel B, c-Rel, NF- κ B 1/p50, and NF- κ B 2/p52). RelA/p65, Rel B, and c-Rel are the transcriptionally active members of this family. The NF- κ B heterodimer, p65/p50, is normally retained in the cytoplasm in association with I κ B α or I κ B β (Chen et al., 2003). In most cancers, the Akt and NF- κ B pathways are constitutively expressed and prevent TRAIL/Apo2L (tumor-necrosis factor-related apoptosis ligand)-induced apoptosis (Sovak et al., 1997; Wang et al., 1999). In

response to chemokine- TRAIL, both MDA-MB-231 and MCF7 breast cancer cell lines have been shown to induce the expression of both RelA/p65 and c-Rel within 60 min and 20 min, respectively. The authors have demonstrated that the dual function of NF- κ B as an inhibitor or an activator of apoptosis depends on the relative levels of RelA/p65 and c-Rel subunits, respectively. The ratio of c-Rel and Rel A/p65 subunits of NF- κ B complex determine the expression of death receptors (DR), DR4 and DR5, and the survival genes that modulate TRAIL-induced apoptosis (Ravi et al., 2001; Chen et al., 2003). Therefore, the absence or a low expression of RelA/p65 with a concomitant increase in c-Rel will activate the apoptotic pathway.

EGCG from green tea has been shown to sensitize human prostate carcinoma LNCaP cells to TRAIL-mediated apoptosis, decrease the levels of matrix-metalloproteinase, and increase the levels of pro-apoptotic proteins, BAK and BAX (Siddiqui et al, 2008). HepG2 cells treated with epicatechin have been shown to inhibit the activation of I κ K- α (Granado-Serrano et al., 2010a) and induce apoptosis via induction of TRAIL (Jacquemin et al., 2010). Majority of the studies with polyphenols have observed a decrease in expression of RelA/p65 by western analysis. These studies have indicated that polyphenols down regulate the activation of NF- κ B by inhibiting its release from the I κ - κ B complex. Unavailability of active NF- κ B results in the inhibition of cell proliferation and induction of the cell cycle arrest that ultimately leads to cell death (Granado-Serrano et al., 2010a; Panza et al., 2011; Benvenuto et al., 2013; Tsumagari et al., 2015). .

In our study, we did not observe any significant effect on the expression of RelA/p65 and Notch1 transcripts (Figure 14). This indicates that aWE exerted its effect by

inducing a (novel) signaling pathway that is independent of Notch1 and NF- κ B signaling. The signaling pathway induced in MDA-MB-231 in response to WE has not been deciphered. Since we see an induction of apoptosis, it will be interesting to discern the expression levels of c-Rel, an apoptosis inducing subunit of NF- κ B in WE- treated MDA-MB-231 cells.

A WE Treatment has No Effect on the Transcription of MXD4 and Negatively Regulates Transcription of Sp1

The cyclin B1 promoter has a number of consensus binding sites for transcription factors (Figure 17). The promoter includes a CCAAT-box binding factor, NF-Y, an E-box binding factors USF, E2A, and c-Myc/MAX, and Sp1 that binds to a GC-rich region 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3' (Cogswell et al., 1995; Porter & Donahue, 2003). There are no RelA/65 binding sites in the promoter of cyclin B1. Repressors of cyclin B1 transcription include p53, p73, and MXD4. Both, p53 and p73, can each bind to Sp1 and modulate its function and cease the transcription of cyclin B1 (Innocente & Lee, 2005a; Innocente & Lee, 2005b). MXD4 protein can displace myc from the myc: MAX complex and can competitively bind to MAX to form a MXD4: MAX complex. This repressor complex can bind to the E-box in the promoter region, thereby repressing the gene transcription.

Transcription factor c-myc is a ubiquitous TF that activates many genes associated with the cell cycle (e.g. Cyclin D1, pRB) and is also involved in activating the genes responsible for apoptosis (Prendergast, 1999). The expression of c-myc is upregulated in many cancers (Bretones et al., 2015). C-myc:MAX heterodimers bind to the E-box sequences in the promoter of the gene and initiate or enhance the transcription of that

gene (Hurlin et al., 1995). When bound to the E-box, the c-myc:MAX heterodimers can enhance the transcription of cyclin B1. In our study, we have analyzed the expression of MXD4, a MAX-dimerization protein 4, in MDA-MB-231 cells treated with a WE.

Figure 17. Schematic representation of the promoter elements of cyclin B1. Arrow represents the transcription start site. NF-Y represents nuclear factor Y, SP1 is a specificity protein 1, AP2 is an activating protein 2, E2A is a transcriptional activating factor, USF is an upstream stimulatory factor, MAX is a myc- associated protein factor X, and MXD4 represents MAX dimerization protein 4.

Repression of transcription of cyclin B1 is possible if MXD4 competitively displaces c-myc from MAX and forms a heterodimer (MXD4: MAX). If bound to the E-box in the promoter of cyclin B1, the MXD4:MAX heterodimeric repressor complex can negatively affect the transcription of cyclin B1. In our study, RT-qPCR data did not demonstrate any significant decrease in MXD4 in the WE-treated MDA-MB-231 cells. This possible interaction of MAX and MXD4 and their binding to the E-box in cyclin B1 promoter can be confirmed by performing the chromatin immunoprecipitation (ChIP) assays in MDA-MB-231 cells treated with a WE.

To understand how cyclin B1 transcription may have been repressed, we chose the transcription factor Sp1 which positively regulates the transcription of cyclin B1. Overexpression of Sp1 is observed in many cancers, and is considered as a negative prognostic factor (Guan et al., 2012; Beishline & Azizkhan-Clifford., 2015; Vizcaino et al., 2015). In normal dividing cells, there is a positive correlation between expression of

cyclins A, B1, D1, and E1, and Ki67 (Kanavaros et al., 2001). Cyclin B1 has two Sp1-binding sites in its promoter region (Figure 17). Sp1 is an activator of cyclin B1 gene. It has been demonstrated that any mutation in the Sp1 binding region represses transcription of cyclin B1 (Innocente & Lee, 2005a; Innocente & Lee, 2005b).

In our study, RT-qPCR analysis on cDNAs made from DMSO and WE-treated MDA-MB-231 cells revealed a 50% decrease in Sp1 transcripts after 1 h treatment with a WE. After analyzing the promoter sequences of cyclins A1, C, D1, E1, H, K, T, and Ki67, we confirmed that they all have Sp1 binding-consensus sequence 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3' in their promoter region. These genes are dependent on the binding of Sp1 in their promoter regions for their transcription. Under normal conditions, Sp1 positively regulates the genes necessary for cell proliferation. All these genes also demonstrate a steep drop in amplifiable transcripts after treatment with a WE. It is possible that the drop in amplifiable transcripts effect is due to the limited number of Sp1 transcripts, and thereby, proteins.

The ATF (activation transcription factor) and CREB (c-AMP reactive element binding protein) complex i.e., ATF/CREB complex, is responsible for the transcription of cyclin A. In a study with polyphenols from red wine, a dose and time-dependent decrease in the transcription of cyclin A was observed in human aortic smooth muscle cells and human umbilical vein endothelial cells (Iijima et al., 2000). In a time-course analysis, a decrease in expression of ATF and CREB transcripts preceded the decrease in cyclin A, demonstrating that polyphenols control the expression of the cell cycle related gene by affecting their transcription factors (Iijima et al., 2000). Does this phenomenon

occur in cells treated with a WE, where Sp1 transcription precedes transcription of Sp1-dependent genes? It remains to be analysed and confirmed.

Sp1 is a ubiquitous transcription factor, with more than 12,000 binding sites within the human genome, and it can positively or negatively regulate the expression of numerous genes. Sp1 is involved in basic transcription as well as in the regulation of a large number of cellular genes. It is also a constitutive activator for most of the housekeeping genes (Pugh et al., 1990; Chu, 2012). Sp1 and Sp1-regulated genes have been shown to be downregulated by curcumin in colon cancer cells (Gandhy et al., 2012; Noratto et al., 2013; Vizcaino et al., 2015).

Both p53 and its paralog, p73, have been shown to modulate the activity of Sp1 bound to the Cyclin B1 promoter and repress transcription of the cyclin B1 gene (Innocente & Lee, 2005a; Innocente & Lee, 2005b). There are, for example, multiple plausible ways Sp1 can affect cell proliferation. Sp1 protein has a total of 164 serine and threonine residues. These residues can be phosphorylated by signaling kinases, for example, extracellular signal regulated kinase 1/2 (ERK1/2), PI(3)K-like, ATM/ATR, Jun-N-terminal kinase (JNK), or DNA-protein kinase (DNA-PK). These kinases cause structural alterations by phosphorylating serine residues on Sp1 protein and plausibly affect its affinity to bind to the DNA, induce its degradation by proteosomal pathway, and/or its ability to trans-activate transcription (Iwahori et al., 2008; Chuang et al., 2008; Spengler et al., 2008; Beishline et al., 2012).

In this study, based on preliminary RT-qPCR analysis, we hypothesize that a WE-treatment possibly affects the transcription of Sp1 as well as the modification of the Sp1

protein. Further studies would aid us to understand, and perhaps explain whether decreases in transcription of Sp1 in response to a WE-treatment lead to a decrease in expression of target genes that have Sp1-binding sites in their promoters. Also the possibilities of Sp1 proteins undergo modification in response to WE-treatment that may affect its interaction with other proteins as well as its ability to promote transcription remain to be explored.

Many unexplored and altered signaling pathways induced by a WE-treatment may result in a decrease in the expression of Sp1 proteins and also modify their function. Sp1 has a potential to be extensively modified, and such a diverse population of Sp1 may be present within one cell. This may result in fewer Sp1 proteins that can bind to the cyclin promoters and activate transcription. As a consequence, there are fewer copies of cyclins such as cyclin C, H, K, and T. Cyclins C, H, K, and T are all responsible for activating RNA pol II at specific steps of transcription. Cyclin C/cdk8 is responsible for forming a mediator complex and phosphorylating CTD domain of the large subunit of RNA Pol II. We have seen that WE affects transcription of cyclin C, resulting in fewer pre-initiation complexes being formed. A limited or reduced pool of the cyclins C, H, K, and T will result in fewer activated RNA Pol II complexes, which may finally culminate in an overall reduction in transcription. A further investigation of a plausible signaling pathway induced in the cells treated with a WE may help understand whether the treatment affects the stability of Sp1 protein (Spengler et al., 2008; Wang et al., 2008). The new study can further determine if the degradation of Sp1 affects the genes that are

dependent on it for their transcriptional activation, e.g., transcription of cyclins and Ki67 studied in this research.

Conclusions

We have demonstrated that WE negatively affected the transcription of all the cyclins, the expression of transcription factor Sp1 and the proliferation of marker Ki67. To our knowledge, this is the first study where a time course analysis was employed to unravel the effect WE inflicted upon transcription of the cyclins and some genes involved in cell proliferation.

Currently, the focus of cancer therapeutics is to either employ a combination treatment with different polyphenols, or polyphenols and other cancer drugs that can lead to more effective antitumor effects, rather than treatment using only one of the compounds (Fantini et al., 2015). Since there are limited successful chemotherapeutics available for TNBC, understanding the effect of WE on cell cycle related proteins at the molecular level will open an attractive possibility of combinatorial therapy with WE and conventional drugs in treating TNBC.

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