RESVERATROL ANALOGS: POTENTIAL CHEMOPREVENTIVE AGENTS IN

BREAST CANCER

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By

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RESVERATROL ANALOGS: POTENTIAL CHEMOPREVENTIVE

AGENTS IN BREAST CANCER

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ABSTRACT

Epidemiological data and studies in rodent models strongly support the role of estrogens in the development of breast cancers. Estrogens have been added to the list of known human carcinogens. Exact mechanisms underlying the initiation and progression of estrogen-related cancers are not clear. Literature evidence and our studies strongly support the role of estrogen metabolism mediated oxidative stress in estrogen-induced breast carcinogenesis. It was recently demonstrated from our laboratory that antioxidants vitamin C or butylated hydroxyanisole (BHA) strongly inhibit 17β-estradiol (E2)-induced breast tumor development in female ACI rats. The objective of present study was to characterize the role of CNC b-zip transcription factors in antioxidant-mediated prevention of breast cancer.

The present study was based on the central hypothesis that 17β-estradiol mediates carcinogenic insult in the cellular environment by producing reactive oxygen species (ROS) / oxidative stress during its metabolism to quinones. This oxidative stress can be controlled by production of phase-II detoxifying antioxidant enzymes. In order to test this hypothesis we examined the cellular levels of different antioxidant enzymes after treatment with E2. We then investigated the molecular mechanism(s) and pathways

involved in E2-induced breast carcinogenesis. To address the question if antioxidant agents can reverse E2-induced oxidative stress and ultimately mediate chemoprevention of breast cancer, we checked several different cellular antioxidant enzymes' levels and dissected out possible molecular pathways involved after treatment with naturally occurring well studied antioxidants like resveratrol (Res), resveratrol analogs (TIMBD and HPIMBD) and Vitamin C.

Resveratrol has been shown to reduce primary tumor growth of xenografts in a nude mouse model. But its clinical applications in prevention of breast cancer are limited because of its lower efficacy in *in vivo* systems. Thus, to improve the anticancer and antioxidant efficacy of Res and to use it as a successful agent targeting breast cancer, pharmacologically active resveratrol analogs have been synthesized. Our newly synthesized Res-analog compounds: 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD) and 4-(E)-{(4-hydroxyphenylimino)-methylbenzene, 1, 2-diol} (HPIMBD), have effectively inhibited the growth of breast cancer cells and have no/minimal cytotoxicity towards normal cells. To further delineate the mechanisms responsible for higher growth inhibitory potency of TIMBD and HPIMBD, we have aimed at finding out its antioxidant potentials. In this current work we have shown the contribution of TIMBD and HPIMBD in providing antioxidant defense in human breast epithelial cells. In our previous studies we have shown that estrogen-induced breast carcinogenesis is initiated by down-regulation of expression of the antioxidant enzymes, superoxide dismutase 3 (SOD3, also known as extracellular superoxide dismutase) and NAD(P)H:Quinone Oxidoreductase 1 (NQO1), via a nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent signaling pathway. In this present study, we have shown that a new and potent

resveratrol analogs TIMBD and HPIMBD, synthesized by our collaborating team functions to inhibit E2-dependent breast cancer development by up-regulation of the expression of SOD3 and NQO1 via this same Nrf2-dependent pathway. We have also provided evidence how TIMBD and HPIMBD mediate antioxidant defense through regulation of CNC-bzip transcription factors other than Nrf2; nuclear factor erythroid 2-related factor 1 (Nrf1) and nuclear factor erythroid 2-related factor 3 (Nrf3).

We also investigated the potential roles of Res-analogs in prevention of epithelial-mesenchymal transition (EMT). An epithelial-mesenchymal transition can be defined as a biological process which mediates a phenotypical change in the polarized epithelial cells to mesenchymal cells. Epithelial cells, which interact with basement membrane, undergo different complex biochemical and molecular changes to become mesenchymal cells. The process of EMT increases a cell's migratory and invasive properties. It is well documented that the process of EMT plays a very critical role in cancer metastasis. The effects of Res-analogs on EMT and the migration of human breast cancer cell lines were studied. We found that Res-analogs significantly increased epithelial marker E-cadherin expression and down-regulated matrix metalloproteases (MMPs) and expression of mesenchymal markers, such as snail, slug, zeb1/2. In present studies, we have demonstrated the potential of Res-analogs in prevention of EMT these studies suggest that our novel Res-analogs may have the potential to be therapeutic agents for breast cancer chemoprevention. In order to dissect out the possible molecular mechanism of Res-analogs on the suppression of EMT and breast cancer cell metastasis, we found a critical involvement of β-catenin. The expression and nuclear translocation of β-catenin

was significantly down-regulated with Res-analogs implicating that these analogs may prevent breast cancer cell metastasis involving β-catenin pathway.

We have also tested the ability of these Res-analogs to inhibit the proliferation of 5 breast cancer cell lines and 3 non-neoplastic breast epithelial cell lines and compared their inhibition potential with Res. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell proliferation was carried out in human breast cancer and non-neoplastic breast epithelial cell lines. The breast cancer cell lines tested were MCF-7, T47D, MDA-MB-231, MDA-MB-468 and BT-20. The non-neoplastic breast cell lines tested were MCF-10A, MCF-10F and HMEC. Of all the analogs tested, two analogs, 4-hydroxyphenyl-immino-methylbenzene-1,2-diol (HPIMBD) and TIMBD showed higher potency for inhibiting the proliferation of breast cancer cells compared to Res. Furthermore, TIMBD or HPIMBD showed higher potency for inhibiting the growth of triple negative breast cancer cells (MDA-MB-231, MDA-MB-468 and BT-20) compared to estrogen receptor positive breast cancer cells MCF-7 and T47D. There was neither inhibition nor proliferation by TIMBD or HPIMBD of non-neoplastic breast epithelial celllines. TIMBD and HPIMBD induced Beclin-1 and LC3-II which suggest autophagy mediated inhibition of cell growth. Beclin-1 is known to be suppressed in breast cancers and it's over expression is reported to inhibit breast cancer. Results from our studies demonstrate that Res-analogs TIMBD and HPIMBD are better than Res in inhibiting specifically breast cancer cell growth and shows higher potency for inhibiting the growth of triple negative breast cancer cells by inducing autophagy with an earlier onset for triple negative breast cancer cells. Therefore, TIMBD and HPIMBD may be better chemotherapeutic agents than Res against breast cancer and more specifically

against triple negative breast cancer cell growth, a cancer type prevalent in minority African American population with poor prognosis.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of School of Graduate Studies have examined the dissertation titled "Resveratrol Analogs: Potential Chemopreventive Agents in Breast Cancer" presented by Anwesha Chatterjee, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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TABLES

ILLUSTRATIONS

ABBREVIATIONS

BHA- Butylated hydroxyanisole

CNC-Cap and Collar

Cyp-Cytochrome P450

DMEM- Dulbecco's modified Eagle's medium

DNA-Deoxyribonucleic acid

DMSO-Dimethylsulfoxide

DCIS-Ductal carcinoma in situ

E2-17β-estradiol

ER-Estrogen receptor

EMT-Epithelial-mesenchymal transition

DCFDA-Dichlorofluoroscein diacetate

HPIMBD-4-(E)-{(4-hydroxyphenylimino)-methylbenzene, 1, 2-diol

HER2-Herceptin Receptor 2

ILC-Invasive lobular carcinoma

LCIS-Lobular carcinoma in situ

LDH-Lactate dehydrogenase

MTT 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl tetrazolium bromide

MMP-Matrix metalloproteases

MFI-Mean Fluorescence Intensity

Nrf1- Nuclear factor erythroid 2-related factor 1

Nrf2- Nuclear factor erythroid 2-related factor 2

Nrf3- Nuclear factor erythroid 2-related factor 3

NQO1- NAD(P)H:Quinone Oxidoreductase 1

8-OHdG-8-hydroxy-2'-deoxyguanosine

PBS -Phosphate buffer saline

PR-Progesterone receptor

Res-Resveratrol

RNA-Ribonucleic acid

ROS -Reactive oxygen species

RT-PCR-Reverse transcriptase-polymerase chain reaction

SOD-Superoxide dismutase

TIMBD-4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol}

TNBC-Triple negative breast cancer

VC-Vitamin C

Wnt-Wingless family of proteins

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DEDICATION

Dedicated to my father who could not live to see his dream come true and to my mother

who made sure that my father's dream come true.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Breast Cancer

Cancer is a group of diseases that is caused due to uncontrolled division and growth of cells in the body. The cells of the body which lose the control over cell-division and growth, due to gene-mutations, become immortal and are called as cancer cells. It is very common property of cancer cells to form an aggregated mass of cells or lump which is defined as tumor. Breast cancer (Figure 1) is a malignant tumor that originates in the breast tissue. If not diagnosed at the early stages, these malignant cancer cells can invade the breast tissue and spread to different other tissues of the body, resulting in deadly consequences of life-threatening metastatic breast cancer. Breast cancer is a disease which occurs mostly in women, but is not limited to; men can also get this disease.
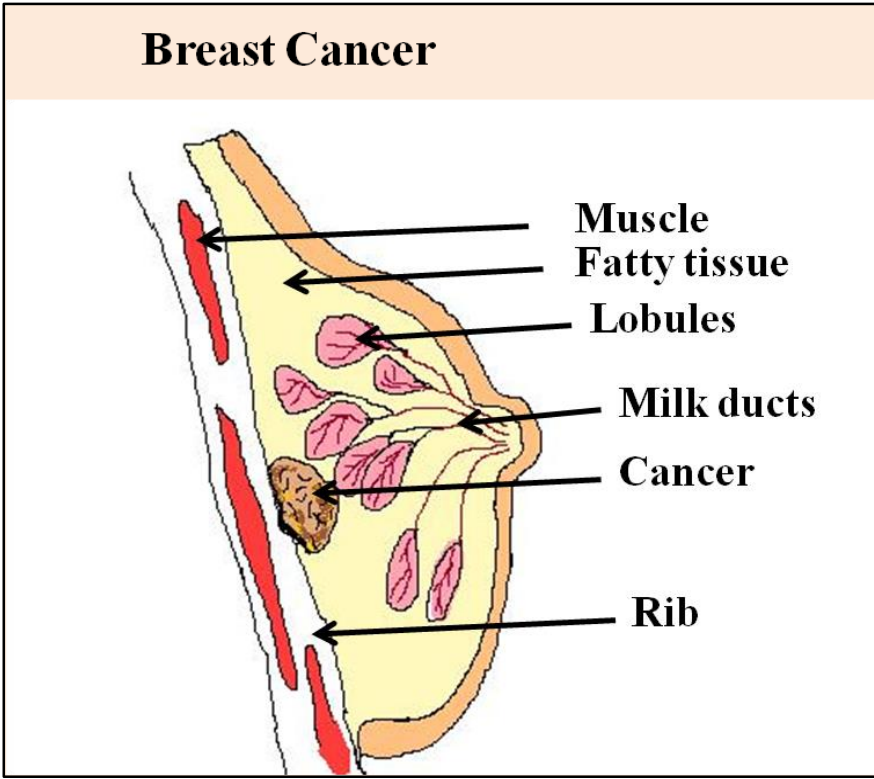
Figure 1 : Breast cancer

The female breast tissue is made up of milk-producing glands or lobules, small tubes or ducts that carry the milk produced in the lobules to the nipple, and fatty, connective and lymphatic tissue which is defined as stroma. The ducts and lobules remain surrounded by the stroma. Most frequently occurring breast cancer is ductal carcinoma which begins in the cells lining the milk ducts. The other type of breast cancer which is found in a small number begins in the milk glands or lobules, called as lobular carcinoma.

1.2. Types of Breast Cancer

Breast cancer can be of several types based on the tissue of origin (ductal or lobular) or based on the nature of the cancer cells, whether they form the malignant lump at only one site or spreads in surrounding other tissues of the body (in situ or invasive, respectively). But, there can be occurrence of such cases where breast cancer can be a combination of different types. The frequently occurring breast cancer types include Ductal carcinoma in situ (DCIS), Invasive ductal carcinoma, Invasive lobular carcinoma (ILC). Although Lobular carcinoma in situ (LCIS) exists, it is not considered as truly malignant type, but considered as a risk factor that can develop into invasive breast cancer. The less frequently occurring breast cancers are inflammatory breast cancer and triple-negative breast cancer. For the convenience of breast cancer prognosis and treatment, specific biological markers have been identified. Based on these molecular markers or hormone receptor status on cell surface breast cancer can be classified as estrogen receptor (ER) positive (ER+) or ER negative (ER-), progesterone receptor (PR) positive (PR+) or PR negative (PR-), and HER2/neu positive (HER2+) or HER2 negative

(HER2-). Cells with none of these receptors are called basal-like or triple negative breast cancer (TNBC).

1.3. Signs and Symptoms of Breast Cancer

There are no typical symptoms of breast cancer when the tumor is small and can be cured easily. For that reason, it is of utmost importance for women to follow guidelines of breast cancer screening for its detection at an early stage. But as a result of ignorance and negligence when breast tumor grows to a palpable size, it clearly looks like a lumpy mass which does not produce any pain. At times breast cancer can cause swelling of lymph nodes under the arm by spreading into it. The less common symptoms of breast cancer are pain in the breast tissue, visible changes of the colour and texture of the breast skin, swelling, puckering or redness of the breast's skin, discharge from the nipple etc.

1.4. Risk factors of Breast Cancer

Risk factors that affect the chance of getting breast cancer in women include age, genetic risk factors, family history, personal history, race and ethnicity, dense breast tissue, menstrual periods, late pregnancy, oral contraceptives, environmental chemicals, smoking, drinking alcohol, obesity, diet, hormone (estrogen) therapy after menopause etc.

1.5.   Endogenous Hormone Estrogen Levels as a Risk Factor in Breast Cancer

Women with higher levels of natural female sex hormone 17β-estradiol (estrogen) in their circulation are associated with increasing the risk of breast cancer almost twice compared to women with lower levels of estrogen. Not only estrogen, but a higher level of testosterone hormone also increases the risk of breast cancer, particularly in premenopausal women. So, it should be taken in consideration that besides environmental factors, we have our own bodily substances like hormones which play critical role as major risk factors for development of breast cancer.

1.6.   Breast Cancer: The Major Health Problem among US Women

The number of breast cancer survivors has increased as a result of an early detection of the disease and improved treatments. But breast cancer is still the leading cause of death occurring from cancer among women in the United States (1-3). According to the epidemiological data provided by the American Cancer Society and the National Cancer Institute, approximately 14.5 million people are living with cancer on January 1, 2014 in the United States. It is also estimated that by January 1, 2024, approximately 19 million Americans will be diagnosed with cancer. The most prevalent cancers found among male cancer survivors are prostate cancer (43%), colorectal cancer (9%), and melanoma (8%). The most common cancer types found among females cancer survivors are breast cancer (41%), uterine cancer (8%), colon and rectum (8%) (Cancer Treatment & Survivorship Facts & Figures 2014-2015). Breast cancer is a devastating disease among women in the United States and worldwide. It is still the most important health problem among women in the United States. It is estimated as of January1, 2014, approximately 3.1 million women are living in the United States with this disease (Cancer Treatment & Survivorship Facts & Figures 2014-2015).

According to Cancer statistics data 2014, roughly 232,670 new cases will be diagnosed with breast cancer and it is also estimated that approximately 40,000 deaths will occur from breast cancer among American women (Figure 2).

Estimated New Cases*

			Males	Females			
Prostate	233,000	27%			Breast	232,670	29%
Lung & bronchus	116,000	14%			Lung & bronchus	108,210	13%
Colorectum	71,830	8%			Colorectum	65,000	8%
Urinary bladder	56,390	7%			Uterine corpus	52,630	6%
Melanoma of the skin	43,890	5%			Thyroid	47,790	6%
Kidney & renal pelvis	39,140	5%			Non-Hodgkin lymphoma	32,530	4%
Non-Hodgkin lymphoma	38,270	4%			Melanoma of the skin	32,210	4%
Oral cavity & pharynx	30,220	4%			Kidney & renal pelvis	24,780	3%
Leukemia	30,100	4%			Pancreas	22,890	3%
Liver & intrahepatic bile duct	24,600	3%			Leukemia	22,280	3%
All Sites	**855,220**	**100%**			**All Sites**	**810,320**	**100%**

Estimated Deaths

			Males	Females			
Lung & bronchus	86,930	28%			Lung & bronchus	72,330	26%
Prostate	29,480	10%			Breast	40,000	15%
Colorectum	26,270	8%			Colorectum	24,040	9%
Pancreas	20,170	7%			Pancreas	19,420	7%
Liver & intrahepatic bile duct	15,870	5%			Ovary	14,270	5%
Leukemia	14,040	5%			Leukemia	10,050	4%
Esophagus	12,450	4%			Uterine corpus	8,590	3%
Urinary bladder	11,170	4%			Non-Hodgkin lymphoma	8,520	3%
Non-Hodgkin lymphoma	10,470	3%			Liver & intrahepatic bile duct	7,130	3%
Kidney & renal pelvis	8,900	3%			Brain & other nervous system	6,230	2%
All Sites	**310,010**	**100%**			**All Sites**	**275,710**	**100%**

Figure 2: Ten leading cancer types for the estimated new cancer cases and deaths by sex. A total of 1,660,290 new cancer cases and 580,350 cancer deaths are projected to occur in the United States in 2014 (Source: Siegel R, Ma J, Zou Z, Jemal A. 2014. Cancer statistics, 2014. CA: a cancer journal for clinicians)

1.7. Estrogen: A Carcinogen

Long term exposure to estrogens has been associated with development of breast cancer (4-19). Estrogens are now enlisted as chemical carcinogens by the United States government (20-22). Chemical carcinogens function by their electrophilicity. Most of the chemical carcinogens (95%) need to undergo metabolic activation to give rise to reactive electrophilic forms that can react covalently with the nucleophilic groups of DNA, RNA and protein (23-26). The other 5% of the chemical carcinogens are direct electrophilic alkylating agents that do not need metabolic activation. Thus the chemical carcinogens are capable of react readily with DNA molecules, resulting in stable mutations that ultimately lead to cancer initiation.

1.7.1. Molecular mechanisms of estrogen carcinogenesis

Two schools of thought exist to explain carcinogenicity of estrogens. One is hormone receptor-dependent pathway and the other is hormone receptor-independent pathway. In the hormone receptor-dependant mechanism, estrogens bind to the estrogen receptor (ER), activate various cellular signaling pathways leading towards cell proliferation (27-33), cancer promotion and progression. Although the hormonal mechanism explains cell proliferation, it cannot explain the event of cancer initiation which is critically dependant on genetic mutation to happen. In the hormone receptor-independent / metabolic mechanism, estrogens undergo redox reaction during its metabolism producing reactive oxygen species (ROS) that can produce irreversible mutations and also genotoxic estrogen metabolite–DNA adducts, resulting in cancer initiation (4-18, 34). The discovery that specific oxidative metabolites of estrogens, catechol estrogen quinones, react with DNA led to and supports the hypothesis that

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estrogens can become endogenous chemical carcinogens by generating the mutations leading to the initiation of cancer (34-37).

1.7.2. Estrogen-induced Breast Cancer: Implication of oxidative stress

In the breast tissue, 17β-estradiol (E2) can be metabolized by specific cytochrome P450 (Cyp) enzymes to catechol estrogens. Two different catechols are produced. Metabolism of E2 by Cyp1A1 gives rise to 2-hydroxyestradiol (2-OHE$_2$) and metabolism by Cyp1B1 gives rise to 4-hydroxyestradiol (4-OHE$_2$). Among these two products, 2-OHE$_2$ is not genotoxic but 4-OHE$_2$ is highly carcinogenic. Genotoxic 4-OHE$_2$ can further undergo oxidative metabolism and produces semi-quinones and highly reactive quinones. These quinones are highly reactive electrophiles having the ability to readily react with DNA forming depurinating quinone-DNA adducts. During the process of redox cycling between catechol estrogens and E2-quinones, reactive oxygen species (ROS) are generated. The oxidative stress that is thus generated during E2 metabolism can damage DNA, RNA, lipid and protein molecules leading to DNA mutation and other cellular damages and ultimately can cause carcinogenic insult to the cells (Figure 3).
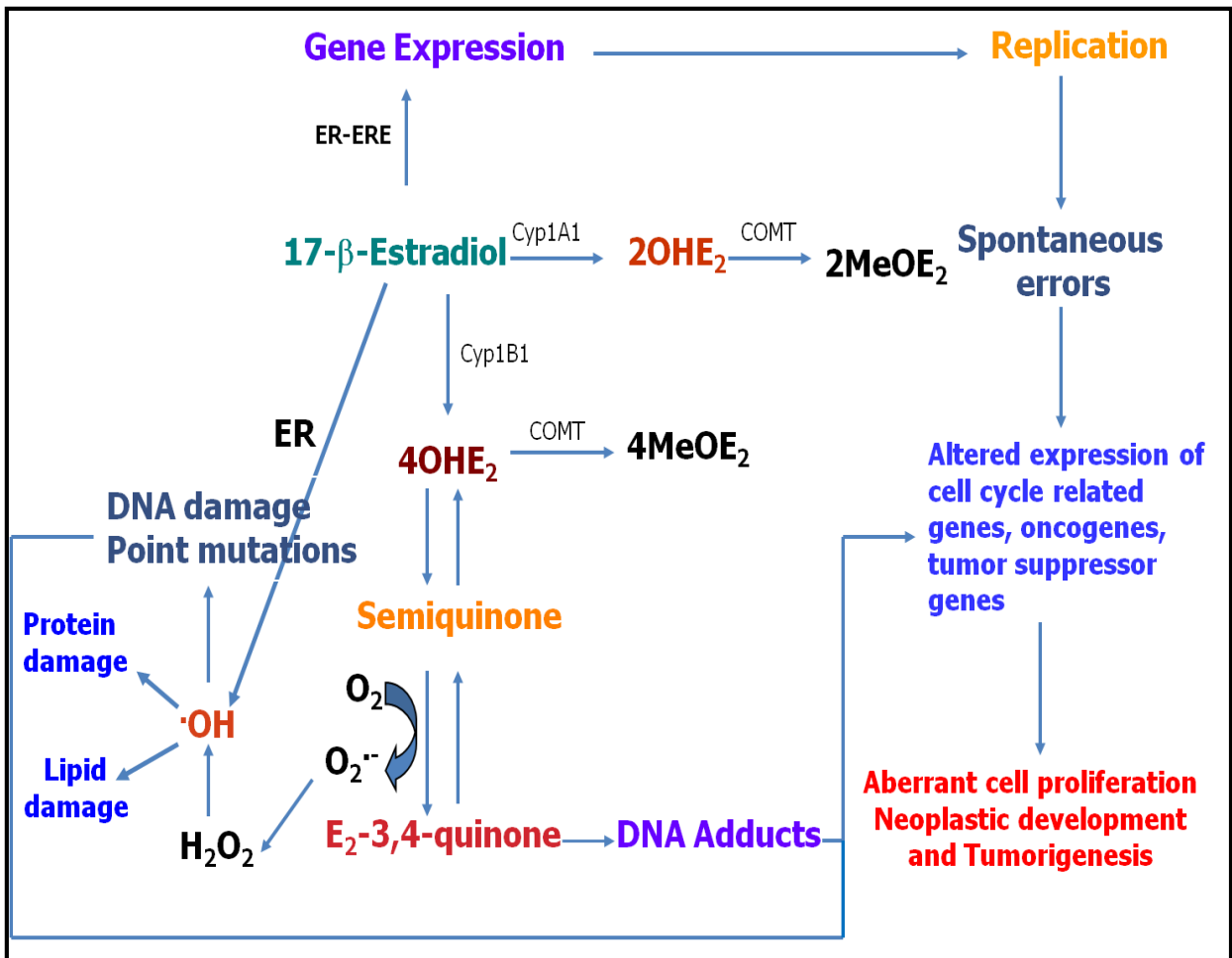
Figure 3: Possible molecular mechanism of E2-induced breast cancer: Role of oxidative stress (Source: Bhat, HK, Oxidative Stress and Disease, 2006)

1.7.3.   Antioxidants in chemoprevention of E2-induced Breast Cancer

Antioxidant can be defined as a molecule capable of inhibition of oxidation of other molecules. Antioxidants can remove free radical intermediates which are generated during oxidation reactions. Antioxidants thus protect cells against free-radical induced toxicity. Antioxidants are widely used in dietary supplements and have been extensively studied for the prevention of cancer and other diseases. Our laboratory has examined the role of prototypic antioxidants vitamin C (VC) (13) and butylated hydroxyanisole (BHA) in E2-induced mammary carcinogenesis (18). Vitamin C is one of the most well known natural antioxidant found in a variety of fruits and vegetables. It has also been used as a dietary supplement in prevention of cancer, cardiovascular and other chronic diseases. Butylated hydroxyanisole is another well studied antioxidant agent extensively used in food additives in order to protect against chemical carcinogens. The anticarcinogenic activity of BHA is attributed to its ability to scavenge free radicals and to induce phase II detoxifying enzymes. In previous studies, our laboratory has shown that antioxidants VC and BHA significantly inhibit E2-induced breast cancer in animal model of breast carcinogenesis, female ACI rats. Antioxidant agents are suggested to reduce breast cancer most likely through induction of antioxidant enzymes or phase II detoxifying enzymes. NAD(P)H-Quinone oxidoreductase 1 (NQO1) is a key antioxidant enzyme involved in defense against reactive oxygen species and protect against carcinogenic insult. NQO1 converts E2-quinones back to E2-catechols, thus making E2-quinones unavailable for reaction with DNA and oxidative stress. The role of NQO1 in prevention of E2-induced breast carcinogenesis has been well studied by our laboratory in an ACI rat

model of breast cancer (36). During metabolism of estrogen, one electron oxidation of catechol estrogens to estrogen quinones take place and superoxide radicals are produced as a result of this redox reacation. These superoxide radicals can get converted to hydrogen peroxide in presence of sufficient superoxide dismutase (SOD) enzymes. Hydrogen peroxide is subsequently removed by cellular catalases and peroxidases. The SODs represent the major cellular defense system against superoxide radicals. In mammalian tissue, three isoforms of SODs have been identified: the cytoplasmic CuZnSOD (SOD1), the mitochondrial MnSOD (SOD2) and the extracellular SOD (EC-SOD or SOD3). Among all SODs, isozyme SOD3 is currently the least studied in relation to hormonal cancer prevention, although recent data support a potentially important role for SOD3 in maintaining oxidative homeostasis in the extracellular matrix, as well as in the nucleus (38-43). Disruption of the *SOD3* gene in mice has been shown to increase their level of sensitivity to environmental stressors (39, 44, 45). Normal mRNA and protein expression levels of SOD3 are relatively low in human lung and breast cancers, as well as in animal models of E2-induced breast cancer (46-48). However, there are relatively few, if any, published reports demonstrating the targeting of SOD3 in prevention of estrogen-dependent breast cancer other than our recently published finding (49). SOD3 is the only member of the SOD family found to date to be suppressed in E2-induced BCs (49). In this study our laboratory has demonstrated the effects of antioxidants VC and BHA on SOD3 expression and role of SOD3 in prevention of E2-induced oxidative DNA damage and breast carcinogenesis. The effects of antioxidants on DNA repair capacity of the cells were very recently explored by our group.

1.8. Phytoestrogens in Chemoprevention of Breast Cancer

According to epidemiologic observations, the incidence of breast cancer is lower in Asian women, who consume significantly higher amounts of phytoestrogens than those of westerners (50). Second- and third-generation descendants of women who migrated to Western countries from Asia have breast cancer risks similar to those of women in the host country, suggesting that lifestyle and not genetic factors explain the low breast cancer risk observed in Asian women (51). However, in spite of recent awareness related to the known chemopreventive properties of phytoestrogens, epidemiologic studies have produced inconsistent results, and the relationship between phytoestrogens and breast cancer remains controversial (52, 53). Moreover, the possible mechanisms of phytoestrogen action in breast cancer have yet to be resolved. Phytochemicals can be categorized into different groups such as chlorophylls, phytosterols, carotenoids, isothiocyanates, phytoestrogens etc (54). Phytoestrogens are one group of nonessential plant chemicals or phytochemicals that have been suggested to play important role in cancer prevention (54-59). Phytoestrogens are bioactive compounds, similar in structure to endogenous estrogens (60, 61). It is evident from literature that higher consumption of phytochemical-rich diets can reduce risk of cancer in both *in vivo* and *in vitro* studies (62-64). Although thousands of different varieties of phytochemicals have been identified, it's still challenging to study millions of more undiscovered plant chemicals and understand their modes of actions. Phytoestrogens are found in various fruits and vegetables, nuts, soy beans and soy products, flaxseeds, dark chocolate, red grapes and red wine (54, 57). Phytoestrogens exert Estrogenic and/or antiestrogenic properties and

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various other biological properties (Figure 4) like antioxidant, antiangiogenic, antiproliferative etc (59, 65-71). It has been studied that postmenopausal breast cancer risk can be reduced following increased consumption of phytoestrogens in the subjects who were without hormone replacement therapy (72).
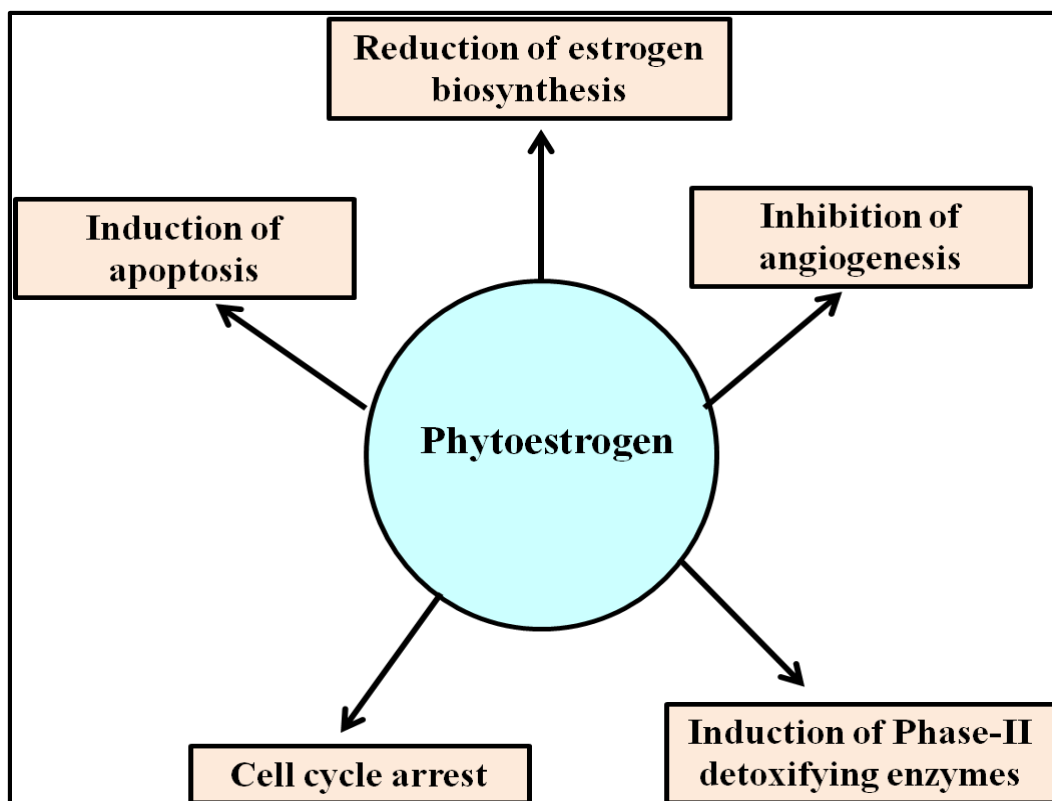
Figure 4: Different potential mechanism of actions of phytoestrogens

1.8.1. Resveratrol and Breast Cancer Prevention

Resveratrol (Res) (3,5,4′-trihydroxystilbene) is a naturally occurring polyphenolic compound from stilbene family (73-76). Resveratrol is considered as a phytoestrogen as it is an estrogen like compound present in plants; it has got structural similarity with the estrogenic compound diethylstilbestrol (77-79). It is found in various plant products grapes, peanuts, berries, Polygonum roots etc (80-83). It can be found as cis- or trans-stereo-isomeric forms (84-86). It was first discovered by Michio Takaoka more than 60 years ago, in the resin of Veratum grandiflorum (false hellebore) (87). Thereafter, Res was also detected in grapevines (Vitis vinifera) in 1977 by Langcake, who found that the compound was synthesized by leaf tissues in response to fungal infection or exposure to ultraviolet light (88). This property classifies Res as a phytoalexin, compounds produced by plants in response to damage. In recent years, research on Res has described several beneficial effects of this compound to human health (Figure 5). Resveratrol has been reported to have both anticarcinogenic and cardioprotective activities, which could be attributed to its antioxidant and anticoagulant properties (89-94). Resveratrol has been reported to be effective in inhibiting platelet aggregation and lipid peroxidation, altering eicosanoid synthesis, modulating lipoprotein metabolism (95-97), and exhibiting vasorelaxing and anti-inflammatory activities (98). For its anticarcinogenic activities, potential mechanisms of Res action have been studied extensively, though there is no clear consensus on the matter. Resveratrol has been reported to inhibit the three major stages of carcinogenesis: initiation, promotion, and progression (99, 100). Anti-initiation activity was indicated by antioxidant and antimutagenic effects and induction of phase II drug-metabolism enzymes (101). Antipromotion activity was indicated by anti-

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inflammatory effects, and inhibition in vitro of cyclooxygenase and hydroperoxidase (101). Antiprogression activity was described as an induction of human promyelocytic leukemia cell differentiation (101). Resveratrol also inhibits the development of preneoplastic lesion in carcinogen-treated mouse mammary glands in culture and inhibits tumorogenesis in a mouse skin cancer model (101). Many *in vitro* studies have addressed the Res activities in breast cancer cells. Resvertrol exhibits an action in both hormone-sensitive and hormone-resistant breast cancer cells. Resveratrol has also been reported to exhibit anti-initiation, antipromotion, and antiprogression activities in breast cancer cells, where these properties seem to be related to regulation of xenobiotic carcinogen metabolism and anti-inflammatory, antiproliferative, and proapoptotic effects. Chemoprevention of carcinogenesis by nontoxic chemical substances is regarded as a promising alternative strategy to therapy for control of human cancer. In recent years, many naturally occurring substances have been shown to protect against experimental carcinogenesis (13, 102-107). Although Res has shown remarkable promise as a potent chemopreventive agent in breast cancer *in vitro*, there is a long way to go for it to be developed as an agent for chemoprevention / treatment of cancer in clinical settings. Continued efforts are needed, especially well-designed preclinical studies in animal models.
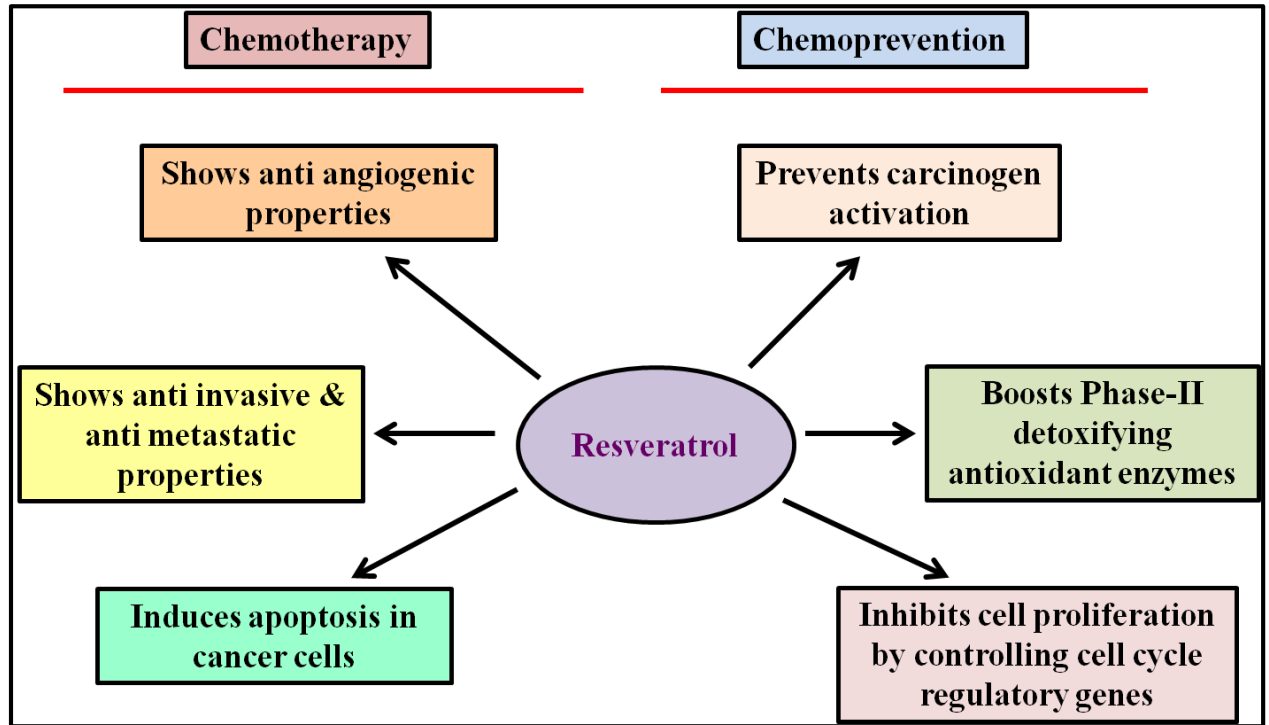
Figure 5: Biochemical mechanisms responsible for chemopreventive and chemotherapeutic potential of resveratrol

1.8.2. Limitations of Resveratrol use in Clinical Settings

A variety of natural compounds are found in nature with unique medicinal values. Resveratrol is one of such hugely studied naturally occurring plant derived phyto-estrogen, well studied for its medicinal properties. It is found in various vegetables, nuts, fruits including grapes and red wine. Res have been well explored for its plethora of medicinal properties in treatment of cancer and a wide range of other diseases (99, 108-114). Resveratrol mediates its anti cancer activity by inducing apoptosis in cancer cells via modulating various signal transduction pathways and targeting different transcription factors responsible for uncontrolled growth of cancer cells (74, 115-119). Not only limited to *in vitro* studies, Res has also been successfully shown to reduce growth of primary tumor xenografts in animal model (120). In spite of its remarkable medicinal properties, Res cannot be used effectively as anticancer agent in clinical settings due to its poor efficacy in human body (121-125). The polyphenol Res is highly absorbed, metabolized and excreted yielding a rather low bioavailability (121-125). Approximately twenty different metabolites of Res have been found in plasma, urine of animals and humans (126-128). The main metabolites of Res found are in the forms of mono- and di-glucuronides (*trans*- and/or *cis*-), mono- and disulfates and sulfoglucuronides (129-131). It has been observed that the plasma concentration of Res-metabolites is much higher than that of parent compound. The highest plasma Cmax of Res-metabolite (967 ng/mL, around 4.2 µM) was reported by Brown *et al.* upon ingestion of 5 g micronized Res in comparison to that obtained (539 ng/mL, around 2.3 µM) after standard Res intake (132).

Therefore, in order to improve the anticancer and antioxidant efficacy of Res and use it successfully against breast cancer and probably other diseases, there is a genuine

need to develop more effective and sensitive analogs of Res. To meet this goal, our collaborative team has synthesized active Res analogs (Figure 6 & Figure 7) (133). In these analogs we have maintained 3,4-dihydroxy substitution on the A ring while varying the substituents at C-4 position on the B ring along with inclusion of aza functionality in the conjugated system (133). The analogs synthesized were named as 3a-f (133). We have studied two of these analogs named TIMBD, 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol; and HPIMBD, 4-(E)-{(4-hydroxyphenylimino)-methylbenzene, 1, 2-diol (133), for their antiproliferative and cytotoxic properties on breast cancer cells. HPIMBD and TIMBD have been shown to inhibit breast cancer cell proliferation effectively and successfully with no cytotoxicity towards normal breast epithelial cells.
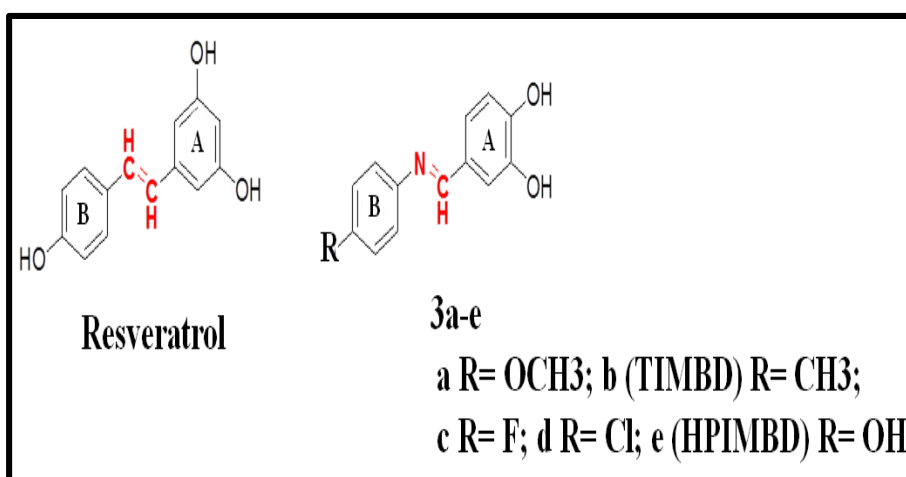
Figure 6: Chemical structure of aza-resveratrol analogs
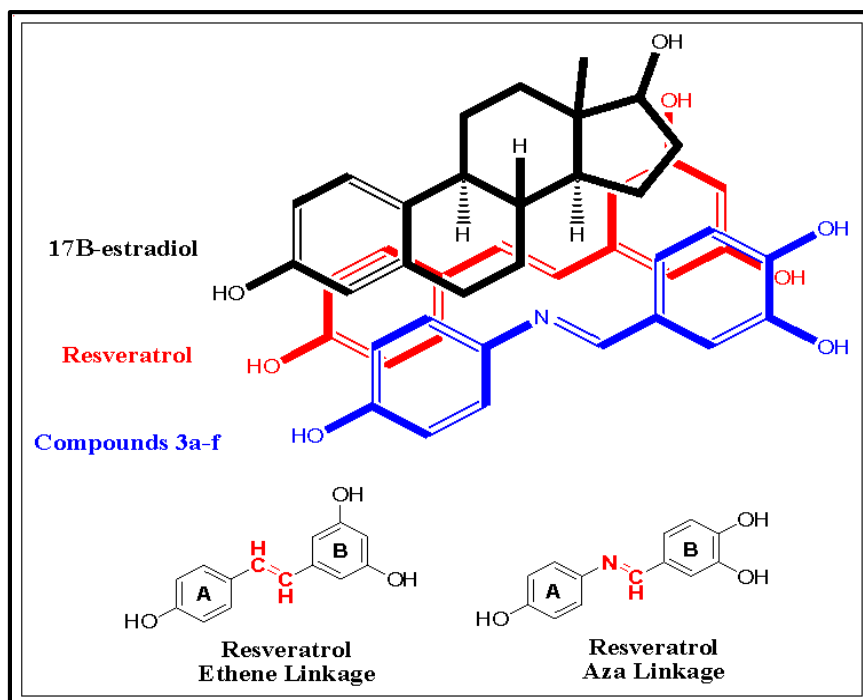
Figure 7: Structural similarities and dissimilarities between resveratrol and Res-analogs

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Cell Culture

All studies were approved by Institutional Biosafety Committee and Institutional Review Board of UMKC. Human breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-7, BT-20 & T47D) and non-tumorigenic breast epithelial cell lines (MCF-10A, HMEC & MCF-10F) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Experiments were performed in passages two to six of cells sub-cultured from a frozen vial. Cells were grown in Dulbecco's modified Eagle's medium/F12 (50:50) media (Mediatech, Herndon,VA) at 37°C in 5% CO_2 environment. Cells were grown to confluence into six-well plates for all experiments. Twenty four hours prior to treatment, cells were washed twice with phosphate-buffered saline (PBS). Breast epithelial cells (MCF-10A, MCF-10F and HMEC) were then grown in phenol red-free Dulbecco's modified Eagle's medium/F12 (50:50), supplemented with 5% charcoal-dextran-stripped horse serum (Cocalico Biologicals, Reamstown, PA). Breast cancer cells (MDA-MB-231, MDA-MB-468, MCF-7, BT-20 & T47D) were grown in phenol red-free Dulbecco's modified Eagle's medium/F12 (50:50), supplemented with 5% charcoal-dextran-stripped fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA).

2.2. Chemicals and Reagents

17 β-Estradiol (E2), Resveratrol (Res) and Vitamin C (VC) were purchased from Sigma–Aldrich (St. Louis, MO). Resveratrol analogs TIMBD and HPIMBD were

provided by our collaborator team (133). 17β-Estradiol, Res and Res-analogs were dissolved in dimethylsulfoxide (DMSO) and vitamin C was dissolved in distilled water prior to treatments. The concentration of DMSO in control experiments or in experimental samples was always 1/1000th (v/v) of the final medium volume. Lipofectamine 2000™ was purchased from Invitrogen Inc. (Carlsbad, CA). Specific siRNAs for Nrf3, Notch 2, β catenin, E-Cadherin and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Treatment of breast cancer cells and non-tumorigenic breast epithelial cells with Resveratrol, Vitamin C, TIMBD and HPIMBD

Human breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-7, BT-20 & T47D) and non-tumorigenic breast epithelial cell line (MCF-10A, HMEC & MCF-10F) were treated with Res, TIMBD or HPIMBD (10-50 μM) for upto 72 hour. MCF-10A cells were also treated with 1 mM vitamin C for up to 72 hours either in the presence or absence of E2. Resveratrol, TIMBD and HPIMBD were dissolved in dimethylsulfoxide (DMSO) prior to treatments. Cells that were treated with vehicle control DMSO (0.1%), were used as appropriate controls. Treated cells were washed with PBS and used for MTT assay, LDH assay, RT-PCR, western blot analyses and other assays according to established methods.

2.4. si RNA Transfection

Small interfering RNAs (siRNAs) for Nrf3, Notch 2, β catenin and control siRNA (scrambled) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MCF-10A cells were transfected with siNrf3 (20 nmol/l) using Lipofectamine 2000^{TM} transfection reagent (Invitrogen, Carlsbad, CA). Scrambled siRNA- (20 nmol/l) transfected MCF-10A

cells were used as a negative control. MDA-MB-231 cells were transfected with siNotch2 (20 nmol/l) and siβ catenin (15 nmol/l) using same transfection reagent. Same concentrations of scrambled siRNAs were used as negative controls for siRNAs for Notch 2 and β-catenin.

2.5. Real time RT-PCR

Real time PCR was used to quantify mRNA expression levels of Nrf1, Nrf2, Nrf3, SOD3, NQO1, β-catenin, Wnt-5a, Wnt-7b and Frizzled-4. Our recently published study suggests that SOD3 and not SOD1 or SOD2 play important role in antioxidant-mediated prevention of E2-induced breast cancer in female ACI rats (49). After different treatments, total RNA from cultured cells was isolated using TRI Reagent (Molecular Research center, Inc.), according to the supplier's protocols. Five μg total RNA was reverse transcribed using the superscript II reverse transcription system and an oligo-dT$_{18}$ primer (Invitrogen, Carlsbad, CA). After reverse transcription, RNase H (2 units/μl) was added to all samples to ensure degradation of the remaining RNA. Real-time PCR was performed in duplicate 25 μl reactions using human-specific Nrf1, Nrf2, Nrf3, SOD3 and NQO1 primers (Qiagen, Valencia, CA and Integrated DNA Technologies, Coralville, IA, USA) using the iCycler iQ5 system (Bio-Rad Laboratories,Hercules, CA). The mRNA expression of cyclophilin, a housekeeping gene, was used for quantification and standardization purposes (134). The expression of the genes under study relative to cyclophilin were determined by dividing the number of c DNA molecules for the gene of interest by the number of cyclophilin c DNA molecules as reported previously (135). Data were analyzed from at least three different samples in each group.

Table 1: List of PCR-Primers and their sequences

Gene	Forward Primer	Reverse Primer
Nrf1	5'-GCT AGT GGA TGG AGA GAC TG-3'	5'-GAA ATG TCT GCT GGA AAC TC-3'
Nrf2	5'-CGG TAT GCA ACA GGA CAT TG-3'	5'- ACT GGT TGG GGT CTT CTG TG-3'
Nrf3	5'-AAG GAG AAC TCA CTT CAG CA-3'	5'-GTG ATG AAA GCA ACT GGA AT-3'
SOD3	5'-TATTCGGGACTCTGAGGGCG-3'	5'-GTCTCACCTTCGCCTTTGCT-3'
NQO1	5'-CCT GGC CCT TGC AAT CTT CTA C-3'	5'-CAG CTC GGT CCA ATC CCT TCA-3'
Frizzled-4	5'-AAC CTC GGC TAC AAC GTG AC-3'	5'-GTT GTG GTC GTT CTG TGG TG-3'
β-Catenin	5'-CAG AAG CTA TTG AAG CTG AGG-3'	5'-TTC CAT CAT GGG GTC CAT AC-3'
Wnt 5a	5'-GGG AGG TTG GCT TGA ACA TA-3'	5'-GAA TGG CAC GCA ATT ACC TT-3'
Wnt 7b	5'- TAT CCC AGA GAG CAA AGT G-3'	5'- GCC TCA TTG TTG TGG AGG TT-3'
Cyclophilin	5'-CCC ACC GTG TTC TTC GAC AT-3'	5'-CCA GTG CTC AGA GCA CGA AA-3'

2.6. Western blotting

Western blot analyses were performed to quantify Nrf1, Nrf2, Nrf3, SOD3, NQO1, MMP2, MMP3, MMP9, E-Cadherin, Snail, Slug, Zeb 1, Zeb 2, Notch-2, β-catenin, Beclin-1 , LC3I/II, Lamin and α-tubulin protein levels. Cell lysates were prepared in RIPA buffer with protease inhibitor cocktail (Sigma–Aldrich, St Louis, MO). Thirty micrograms of total protein isolated from quadruplicates of control or treated cells were size fractionated on a 12% SDS-polyacrylamide gel, and transferred onto PVDF membranes under standard conditions (36, 49). Membranes were blocked in 5% dry non-fat milk/PBS/0.5% Tween-20 at room temperature for 2 hours. Affinity purified polyclonal antibodies against Nrf1, Nrf2, Nrf3, SOD3, NQO1, MMP2, MMP3, MMP9, E-Cadherin, Snail, Slug, Zeb 1, Zeb 2 (Santa Cruz Biotechnology, Santa Cruz, CA), Lamin, Notch-2, β-catenin, Beclin-1 and LC3I/II (Cell signaling Technology) were diluted 1:1500 in PBS/0.05% Tween-20 and used for immunodetection. After incubation overnight at room temperature with the primary antibody, membranes were washed four times for 8 min per wash using PBS/0.05%Tween-20. Horse radish peroxidase conjugated IgG was diluted 1:2000 in PBS/0.5% Tween-20 and used as a secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation for 2 hours at room temperature, the membrane was washed again as described above. Chemiluminescent detection was performed using the BM Chemiluminescence Detection kit (Roche, Indianapolis, IN) and Alpha Innotech FluorChem HD2 (Alpha Innotech, San Leandro, CA) gel documentation system. Membranes probed for the above mentioned specific primary antibodies, were then washed in PBS/0.5% Tween-20 and re-incubated overnight at room temperature with α-tubulin monoclonal mouse antibody (Santa Cruz

Biotechnology, Santa Cruz, CA) diluted 1:2000 in PBS/0.05%Tween-20. Horse radish peroxidase-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology) was diluted 1:2500 in PBS/0.05% Tween-20 and used as a secondary antibody for α-Tubulin detection. Secondary antibody was incubated with the membrane for 2 hours at room temperature prior to chemiluminescent detection using the method described above.

2.7. Colony Formation assay

For clonogenic cell survival assay about 500, each of MDA-MB-231 or T47D cells were treated with 5 μM, 10 μM and 25 μM of Res, HPIMBD and TIMBD for 48 hour. About 500 viable MDA-MB-231 cells were transiently transfected with siRNAs for β catenin (30 nmol/l) and scrambled siRNA (30 nmol/l) for 48 hour (or untransfected MDA-MB-231 cells) were seeded in six-well plates and allowed to grow for 48 hours in phenol red-free complete media. The cells were then washed in PBS and incubated for additional 8 days in complete medium. The colonies obtained were washed with PBS and fixed in 10% formalin for 10 min and again washed twice with PBS followed by staining with crystal violet (0.1%w/v solution in 10% ethanol). The colonies were counted, photographed and compared with respective untreated cells. Each treatment was done in triplicate.

2.8. Mammosphere Formation assay

Mammosphere formation assay was carried out in ultra-low attachment plates (Corning, Lowell, MA). Briefly, 5000 viable T47D cells were seeded in ultra-low attachment plates. After 24 hours, cells were treated with 50 μM of Res, HPIMBD and TIMBD and were grown in serum-free DMEM/ F12 (50:50) medium supplemented with 1x B27 supplement (Invitrogen), 20 ng/ml epidermal growth factor (Invitrogen), 20

ng/ml basic fibroblast growth factor (Invitrogen), 1 μg/ml hydrocortisone (BD Biosciences, Bedford, MA), 5 μg/ml insulin (Invitrogen), 0.1% penicillin/streptomycin (Lonza, Walkersville, MD) and 4 μg/ml heparin calcium salt (Thermo Scientific) at 37°C under 5% CO_2. After 6–8 days of incubation, mammospheres were viewed under the microscope and photographed. Three replicate wells from a 24-well plate were used for each experimental condition.

2.9. Wound Healing assay

Cell migration assay was used to study the metastatic potential of the cells. Briefly, confluent monolayers of MDA-MB-231 cells were treated with Res, HPIMBD and TIMBD at 50 μM doses for 24 hour. A scratch was made through the monolayer, using a standard 200 μl plastic pipette tip, washed twice with PBS and replaced in phenol red-free complete media. Cells migrate into the scratch area as single cells from the confluent sides. After 24 hour, the width of the scratch gap was viewed/measured under the microscope and photographed. Three replicate wells from a six-well plate were used for each experimental condition.

2.10. Measurement of reactive oxygen species (ROS) production

The production of ROS was measured by flow cytometry using dichlorofluoroscein diacetate (DCFDA; Invitrogen). Briefly, MCF-10A cells were treated with E2 (50 nM), Res (50 μM), TIMBD (50 μM) or HPIMBD (50 μM) using serum-free medium at different times in a 6-well plate. After treatment, the cells were washed with PBS and incubated with 10 μM DCFDA in PBS for 30 min at 37°C. 1 mM H_2O_2 treated cells, for 30 min were used as a positive control. After treatment, the cells were harvested and dissolved in 1 ml PBS with 1% FBS to measure the DCF emission at 525 ± 20 nm by

flow cytometer (BD Biosciences, San Jose, CA), and Mean Fluorescence Intensity (MFI) was measured and analyzed.

2.11. 8-OH-dG formation assay

8-hydroxy-2'-deoxyguanosine (8-OHdG), an oxidative stress-mediated DNA damage marker was measured in MCF-10A cells treated with Res, TIMBD or HPIMBD in presence or absence of E2 for 48 hour. DNA was isolated from the cells after all the treatment. DNA was isolated using DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the supplier's protocols with some modifications. To avoid possible spurious DNA oxidation diethylenetriamine pentaacetic acid (0.1 mM) and ascorbic acid (2 mM) were used throughout the DNA isolation process. DNase-free RNase (Sigma-Aldrich, St. Louis, MO) was used to degrade RNA as per supplier's recommendations. The RNA-free DNA was used to estimate 8-OHdG levels using Oxiselect oxidative DNA damage ELISA-kit (Cell Biolabs, San Diego, CA) according to supplier's protocols with some modifications. Briefly, 20 μg DNA was digested with 10 U DNase 1 (Qiagen, Valencia, CA) at 37°C for 30 min in presence of 100 mM $MgCl_2$ and 1 M Tris-HCl (pH 7.4). After that, pH of digested DNA was adjusted to 5.2 with 3 M sodium acetate (pH 5.2). DNA reaction mixture was subjected to 1 μl of nuclease P1 (1 U/μl) digestion for 1.5 hour at 37°C. After incubation, 1 M Tris-HCl (pH 8.0) was used to bring the pH back to 7.4, followed by treatment with 1 μl of alkaline phosphatase (1 U/μl stock) for 1 hour. The reaction mixture was then subjected to 1 μl of each phosphodiesterase I (0.01 U/μl) and phosphodiesterase II (0.01 U/μl) digestion for 30 min at 37°C. The reaction mixture was centrifuged for 5 min at 6000g and the supernatant was used for 8-OHdG ELISA assay.

2.12. Cell survival assay (MTT assay)

The effects of Res, TIMBD and HPIMBD on cell viability were measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The breast cancer cell lines tested were MCF-7, T47D, MDA-MB-231, MDA-MB-468 and BT-20. The non-neoplastic breast cell lines tested were MCF-10A, MCF-10F and HMEC. Briefly, 2000 cells were seeded in 96-well plates and treated with increasing dosages (6.25 µM, 12.5 µM, 25 µM, 50 µM and 100 µM) of Res, TIMBD or HPIMBD for 72 hour. 0.5 mM hydrogen peroxide (H_2O_2) was used as positive control and 10% dimethyl sulfoxide (DMSO) as negative control. After 96 hour treatment was terminated, the medium was replaced with 100 µl of 1mg/ml MTT reagent and incubated at 37°C for 3.5 hour. The medium was removed carefully and purple colour formazan crystals were dissolved in 200 µl DMSO containing 25 µl of Sorenson's glycein buffer by gentle shaking on a shaker. The color formation was measured using Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA) with absorbance at 570 nm and reference at 650 nm.

2.13. Cell survival assay (LDH assay)

The tests of LDH release were conducted using Pierce LDH Cytotoxicity Assay Kit (# 88953). The breast cancer cell lines tested were MCF-7, T47D, MDA-MB-231, MDA-MB-468 and BT-20. The non-neoplastic breast cell lines tested were MCF-10A, MCF-10F and HMEC. All the cells were plated in a 96-well plate (10,000 cells/well) in Dulbecco's modified Eagle's medium/F12 (50:50) media (Mediatech, Herndon,VA) supplemented with fetal bovine serum and incubated overnight at 37°C in 5% CO_2 environment. Next day cells were treated with increasing concentrations (6.25 µM, 12.5

µM, 25 µM, 50 µM and 100 µM) of Res, TIMBD or HPIMBD for 72 hour. Following treatment with compounds, 50 µl supernatant from each well was transferred into a new 96-well plate, 50 µl of reaction mixture from the assay kit was added to each well of the microplate. After incubation at room temperature for 30 minutes, 50 µl of stop solution was added to each well to stop the reaction. Activity of LDH was determined by spectrophotometric absorbance at 490 nm and 680 nm. Cell viability was measured in replicate wells and each set of experiment was carried out at a minimum of three times.

2.14. Caspase-3/7 activity assay

The caspase-3/7 activities were measured using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, MDA-MB-231 and T47D cells were seeded in a 96-well flat bottom plates (Corning, Lowell, MA). Cells were treated with 50 µl of Res, TIMBD or HPIMBD for 24 hour. After treatment cells were lysed with lysis buffer which contained substrate for caspase (Z-DEVD-R110). Cells were incubated with lysis buffer for 6 hour at room temperature. Finally, Caspase activity was determined by measuring the fluorescence at 485/535 nm wavelengths.

2.15. Autophagosome staining with the FITC-fluorescent MAP-LC3 antibody

Treated or untreated MDA-MB-231 cells (with 25 µM of Res, TIMBD or HPIMBD for 12 hour) were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.3% Triton X-100 and blocked with 2% BSA for 30 min. Then cells were incubated with primary antibodies against LC3 overnight at 4°C, washed with PBS, and incubated for another 60 min with an Alexa Fluor®-conjugated antibody (Invitrogen). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma).

A Zeiss LSM 710 confocal microscope system (Carl Zeiss, Germany) was used for colocalization analysis.

2.16. Co-Immunoprecipitation (Co-Ip) Assay

A Pierce kit (Pierce Biotechnology, Inc., Rockford, IL; Catalog #26149) was used for co-immunoprecipitation of interacting proteins, Nrf2 and Nrf3. After direct covalent immobilization of the primary antibody (Nrf2 antibody, Santa Cruz, CA) to amino link plus resin, immunoprecipitation of the Nrf2 protein (bait protein), and co-immunoprecipitation of interacting Nrf3 protein (prey protein) were performed using spin columns. The experiments were repeated reversing bait and prey proteins. Now Nrf3 antibody (Santa Cruz, CA) was covalently immobilized to the resin and immunoprecipitated, the blot was probed to detect co-immunoprecipitation of Nrf2 as prey protein. Unspecific interactions were identified by using the provided control gel and substituting IgG for the specific antibody. Eluted fractions were then subjected to western blotting for identification of the interacting proteins.

2.17. Statistical analysis

Statistical analyses were performed by using Sigma Plot 11.0 (Systat Software, San Jose, CA) and IBM SPSS Statistics 19 software (IBM Inc, Armonk, NY). All cell culture treatments were done in quadruplicate. The Student's *t*-test analysis was used to calculate p values for the comparison between two groups. One-way analysis of variance (ANOVA) and least significant difference (LSD) *post hoc* analyses were used to calculate p values for comparisons among all groups. A p value of ≤ 0.05 was considered significant as shown by '*' or '#'.

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF RESVERATROL ANALOGS

IN INHIBITING BREAST CANCER CELL GROWTH AND POSSIBLE

MECHANISM OF ACTION OF CHEMOPREVENTION

3.1. Introduction

Breast cancer is one of the leading causes of deaths among women in the United States (1-3). Most breast cancers are estrogen-dependent and the contribution of estrogens in development of breast cancer is well accepted (4-19). As a result, estrogens have been added to the list of known human carcinogens (20-22). Apart from estrogen-receptor dependent pathways, estrogen-metabolism mediated oxidative stress-associated pathways are implicated in the development of breast cancer (12, 13, 15). These estrogen-receptor independent, oxidative stress-associated pathways can be successfully targeted for chemoprevention of breast cancer. Our aim is to develop potentially effective pharmacological agents against breast cancer development by targeting estrogen-induced oxidative stress pathways.

The fact that oxidative stress plays an important role in breast cancer development (4, 5) has encouraged people to have antioxidant-rich foods in their daily diets. In estrogen-induced carcinogenesis, metabolic activation of estrogen produces oxidative stress during redox cycling between estrogenic catechols and quinones (15, 18). This oxidative stress can damage DNA, proteins and lipids (7, 19). These damages can modify expression levels of cellular phase-II detoxifying enzymes (36, 37). Expression of cell

cycle regulatory genes can also change as a result of oxidative stress leading to abnormal cell proliferation (37). All these cellular events collectively can ultimately lead to breast tumor development.

The use of prototypic naturally occurring antioxidant vitamin C in cancer chemoprevention and treatment has been reported extensively in the literature (13, 120, 136-138). The mechanism of breast cancer chemoprevention by antioxidants is not well understood and is the focus of our present studies. We have characterized in our studies the antioxidant mechanism of the naturally occurring antioxidants, Res, VC; and Res-analogs, TIMBD and HPIMBD.

Resveratrol is an extensively studied phytoestrogen found in grapes, nuts, red wine and various plants, well known for its protective role against various diseases including cancer (89-94). Amongst its multiple activities, anticancer activity, by targeting cancer cells and induction of apoptosis in cancer cells is an important one (74, 115-119). Resveratrol has been shown to modulate several signaling kinases, transcription factors and other targets leading to cancer cell death (115-119). But Res is not effective as anticancer agent *in vivo*. Its clinical application is limited because of its poor efficacy in human body (121-125). Thus, to improve anticancer and antioxidant efficacy Res and to use it as a successful agent targeting breast cancer, pharmacologically active resveratrol analogs have been synthesized. These Res-analogs have been synthesized in such a way that they are predicted to have better stability than that of the parent compound, Res and have been tested for their ability to inhibit growth of breast cancer cells.

Our newly synthesized aza Res-analog compounds HPIMBD, 4-(E)-{(4-hydroxyphenylimino)-methylbenzene, 1,2-diol} and TIMBD, 4-(E)-{(p-tolylimino)-

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methylbenzene-1,2-diol}(133), have effectively inhibited the growth of breast cancer cells and have no/minimal toxicity towards normal cells (133). To further delineate the mechanisms responsible for higher anti cancer potency of HPIMBD and TIMBD, we have aimed at finding out its antioxidant potentials.

In this current work we have also shown the contribution of HPIMBD and TIMBD in providing antioxidant defense in human breast epithelial cells. In our previous studies we have shown that estrogen-induced breast carcinogenesis is initiated by down-regulation of expression of the antioxidant enzymes, superoxide dismutase 3 (SOD3, also known as extracellular superoxide dismutase) (49) and NAD(P)H-quinone oxidoreductase 1 (NQO1) (36). In this present study, we have shown that our novel and potent resveratrol analogs HPIMBD and TIMBD, synthesized by our collaborating team (133), function to inhibit E2-dependent breast cancer development by up-regulation of the expression of SOD3 and NQO1. We have also provided evidence how these analogs mediate antioxidant defense through down-regulation of cellular reactive oxygen species (ROS) generation and 8-OHdG formation.

3.2. Results

3.2.1. Res-analogs TIMBD and HPIMBD inhibit proliferation in breast cancer cells better than resveratrol and have no effect on non-tumorigenic breast epithelial cells.

The newly synthesized Res-analogs were tested and compared with Res for their antiproliferative effects using MTT assay, using five different breast cancer cell lines and three non-tumorigenic breast epithelial cell lines (Figures 8-15). The breast cancer cell lines tested were MCF-7, T47D, MDA-MB-231, MDA-MB-468 and BT-20 (Figures 8-12). The non-neoplastic breast cell lines tested are MCF-10A, MCF-10F and HMEC (Figures 13-15). MDA-MB-231, MDA-MB-468 and BT-20 are triple negative breast cancer cell lines; MCF-7 and T47D are estrogen receptor positive human breast cancer cell lines. All compounds exhibited antiproliferative effects against all five breast cancer cell lines (Figures 8-12) at 50 µM concentration. Compounds HPIMBD and TIMBD exhibited most potent antiproliferative activity against all breast cancer cell lines compared to Res. The compounds HPIMBD and TIMBD exhibited lower IC_{50} values compared to Res against all five breast cancer cell lines tested. HPIMBD showed IC_{50} values of 4.8, 29, 44, 41, 32 µM and TIMBD showed IC_{50} values of 3.62, 21, 32, 38, 31 µM against MDA-MB-468, MDA-MB-231, T47D, MCF-7 and BT-20 breast cancer cell lines, respectively as compared to Res with IC_{50} values of 41, 66, 76, 77 and 99 µM, respectively (Table 2). None of the newly synthesized resveratrol analogs exhibited any cytotoxic effects against MCF-10A, MCF-10F and HMEC cells at 50 µM concentrations (Figures 13-15) suggesting they are non-cytotoxic towards normal breast cells and selectivity to cancer cells.

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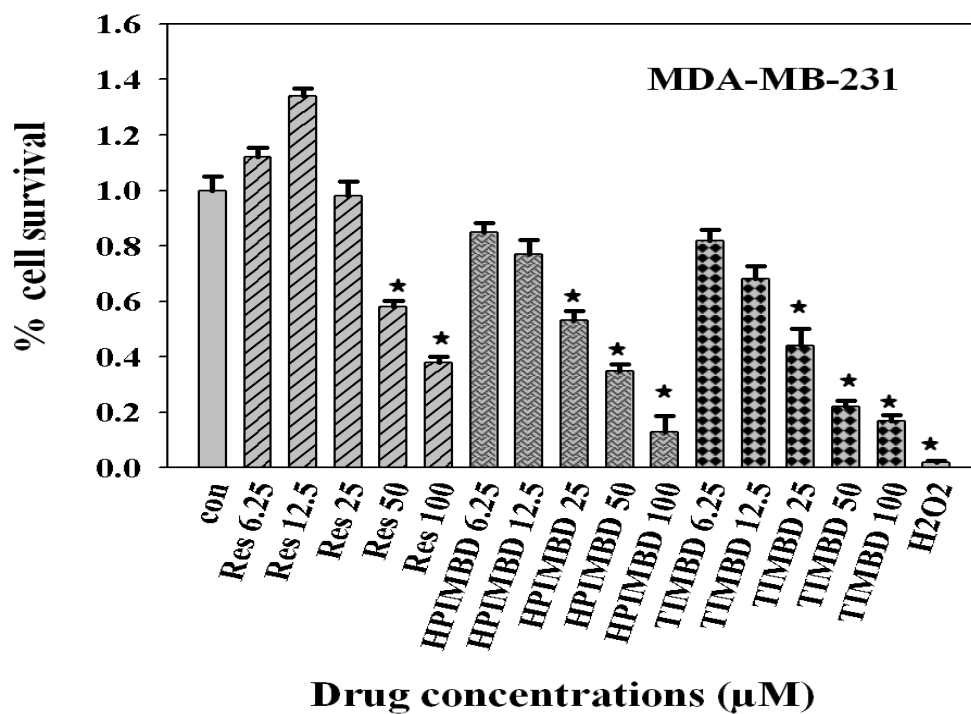
Figure 8: Antiproliferative activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human triple negative breast cancer cells, MDA-MB-231. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for up to 72 hours and were assessed for cytotoxicity by MTT assay. 1mM H2O2 was used as positive control.

* P≤ 0.05 compared to control

Figure 9: Antiproliferative activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human triple negative breast cancer cells, MDA-MB-468. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by MTT assay. 1mM H2O2 was used as positive control.

* $P \leq 0.05$ compared to control
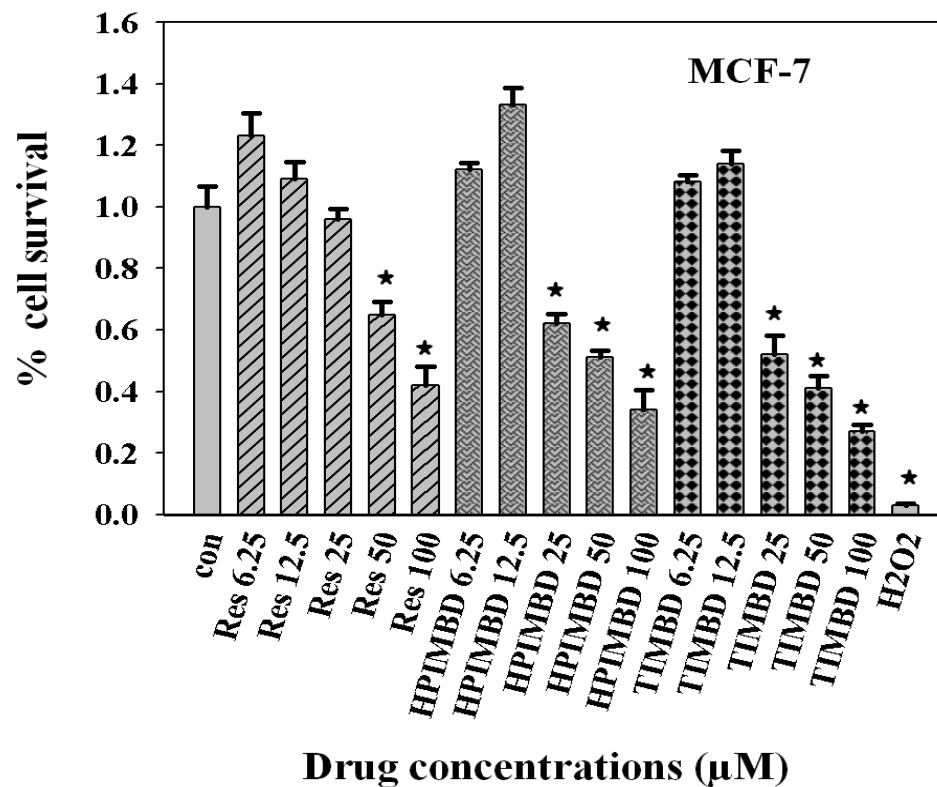
Figure 10: Antiproliferative activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human breast cancer cells, MCF-7. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by MTT assay. 1mM H2O2 was used as positive control.

* P≤ 0.05 compared to control
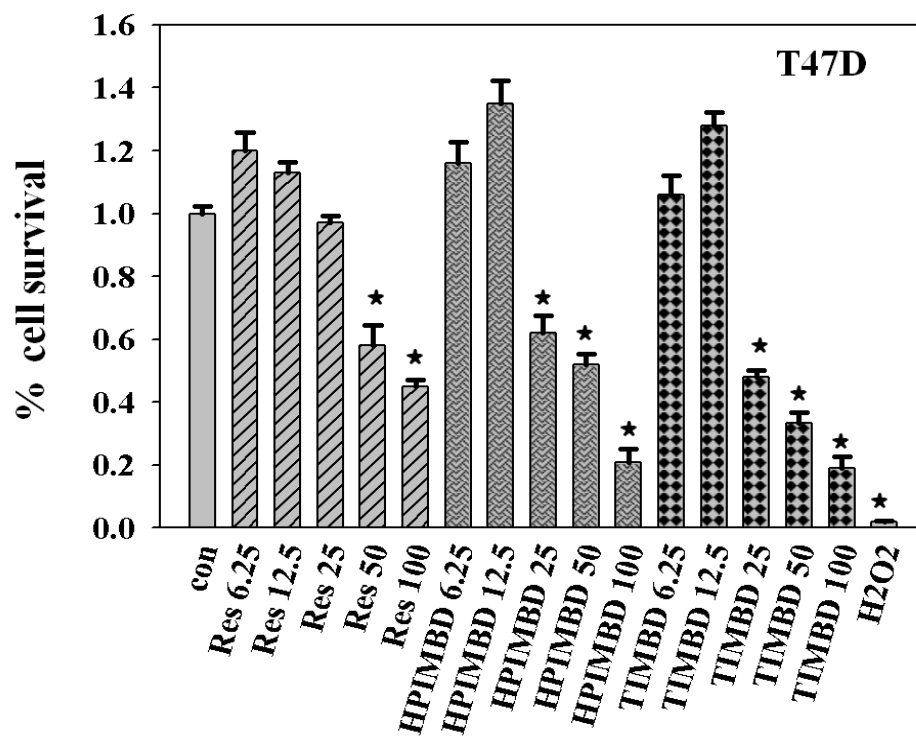
Figure 11: Antiproliferative activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human breast cancer cells, T47D. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by MTT assay. 1mM H2O2 was used as positive control.

* P≤ 0.05 compared to control

Figure 12: Antiproliferative activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human triple negative breast cancer cells, BT-20. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by MTT assay. 1mM H2O2 was used as positive control.

* P≤ 0.05 compared to control
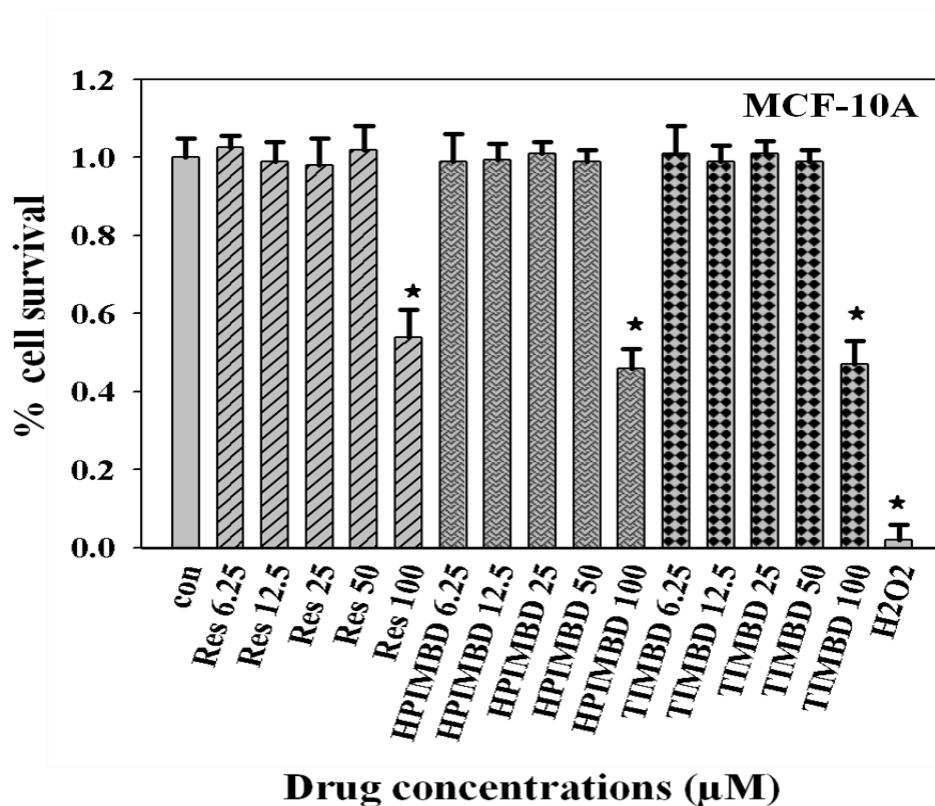
Figure 13: Antiproliferative activity of Aza-resveratrol analogs, HPIMBD and TIMBD against MCF-10A, non-neoplastic human breast epithelial cells. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by MTT assay. 1mM H_2O_2 was used as positive control.

* P≤ 0.05 compared to control
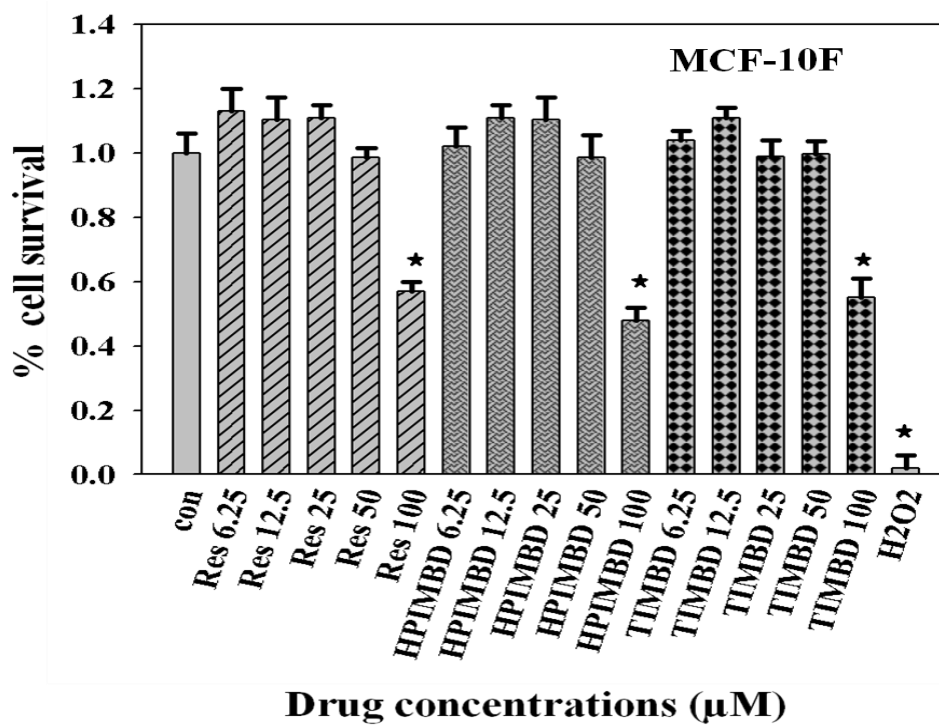
Figure 14: Antiproliferative activity of Aza-resveratrol analogs, HPIMBD and TIMBD against MCF-10F, non-neoplastic human breast epithelial cells. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by MTT assay. 1mM H_2O_2 was used as positive control.

* P≤ 0.05 compared to control

Figure 15: Antiproliferative activity of Aza-resveratrol analogs, HPIMBD and TIMBD against HMEC, non-neoplastic human breast epithelial cells. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by MTT assay. 1mM H_2O_2 was used as positive control.

* P≤ 0.05 compared to control

Table 2. The IC_{50} for different breast cell lines treated with Res, HPIMBD or TIMBD. HPIMBD and TIMBD have better IC_{50} than resveratrol (Res) against breast cancer cell lines; HPIMBD and TIMBD have no effect on non-tumorigenic human breast epithelial cells

Cell line	IC50 (μM) Res	IC50 (μM) HPIMBD	IC50 (μM) TIMBD
MDA-MB-468	41	4.8	3.62
MDA-MB-231	66	29	21
T47D	76	44	32
MCF-7	77	41	38
BT-20	99	32	31
MCF-10A, MCF-10F, HMEC	No effect	No effect	No effect

3.2.2 Res-analogs TIMBD and HPIMBD induce cytotoxicity in breast cancer cells better than resveratrol and have no effect on non-tumorigenic breast epithelial cells.

Breast cancer cell lines MCF-7, T47D, MDA-MB-231, MDA-MB-468 and BT-20 were treated with different doses of TIMBD and HPIMBD (0, 6.25, 12.5, 25 and 50μM) and 50 µM Res for upto 72 hour (Figures 16-22). The extent of cell death was measured 72 hour of treatment by LDH release assays. The amount of LDH released from dying cells is directly proportional to cell death. HPIMBD at 50 µM dose significantly caused about 32 % and TIMBD at 50 µM dose significantly caused about 35 % increase in LDH release in MCF-7 and T47D cells (Figures 18 & 19). HPIMBD at 50 µM dose significantly caused about 38 % and TIMBD at 50 µM dose significantly caused about 40 % increase in LDH release in triple negative breast cancer cells; MDA-MB-231, MDA-MB-468 and BT-20. (Figures 16, 17 & 20). 50 µM of Res treatment for 72 hour caused about 22 % increase in LDH release in MCF-7 and T47D cells (Figures 18 & 19) and about 25 % increase in MDA-MB-231, MDA-MB-468 and BT-20 cells (Figures 16, 17 & 20). To examine the cytotoxic effect of TIMBD and HPIMBD on non-tumorigenic human breast epithelial cells, MCF-10A and MCF-10F cells were treated with above mentioned doses of TIMBD and HPIMBD. LDH release was measured in these cells after 72 hour. TIMBD and HPIMBD did not show any cytotoxic effect on non-tumorigenic breast epithelial cells treated with upto a dose of 50 µM (Figures 21 & 22). From these results in can be suggested that Res-analogs TIMBD and HPIMBD can significantly induce cytotoxic effect in a dose-dependent manner and upto a dose of 50 µM, they do not induce any cytotoxic effect towards non-tumorigenic breast epithelial cells.
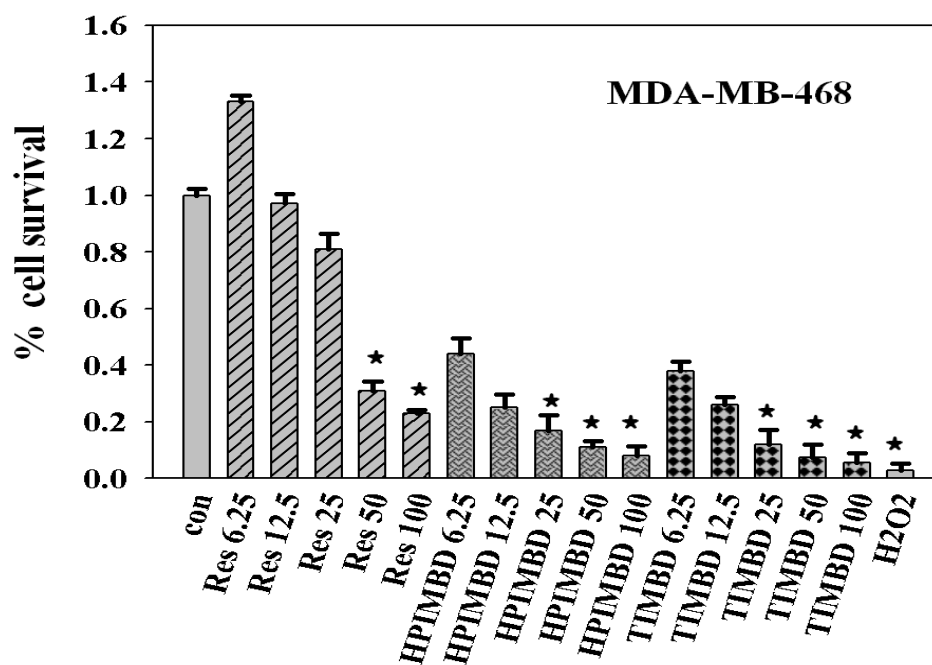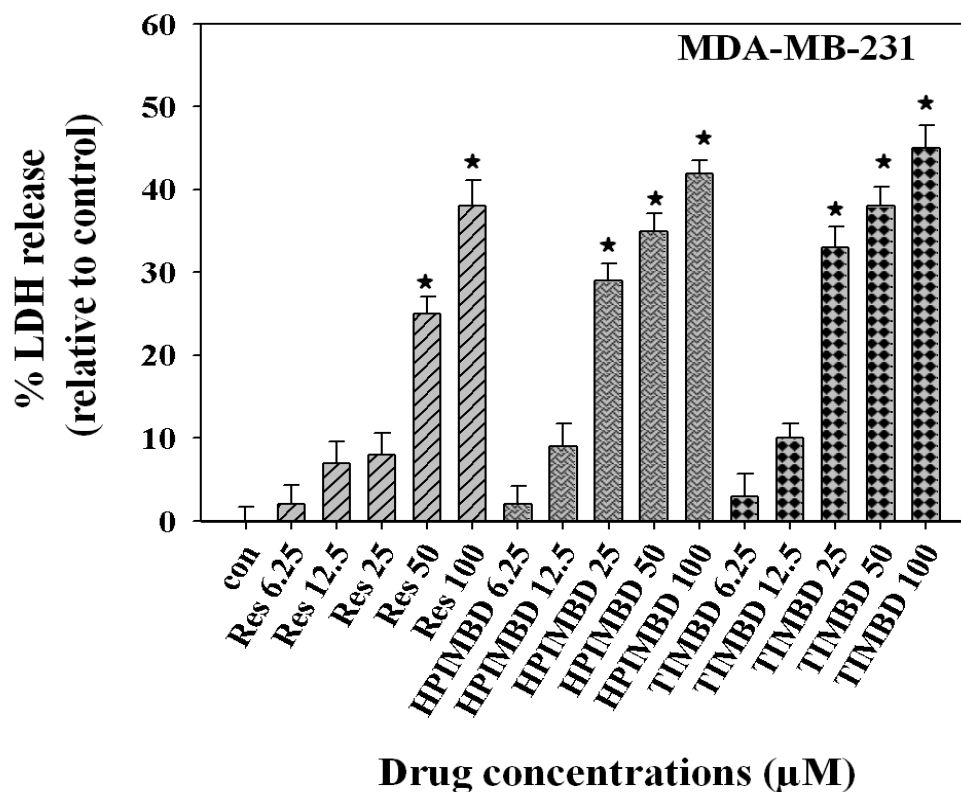
Figure 16: Cytotoxic activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human triple negative breast cancer cells, MDA-MB-231. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by LDH assay.

* P≤ 0.05 compared to control
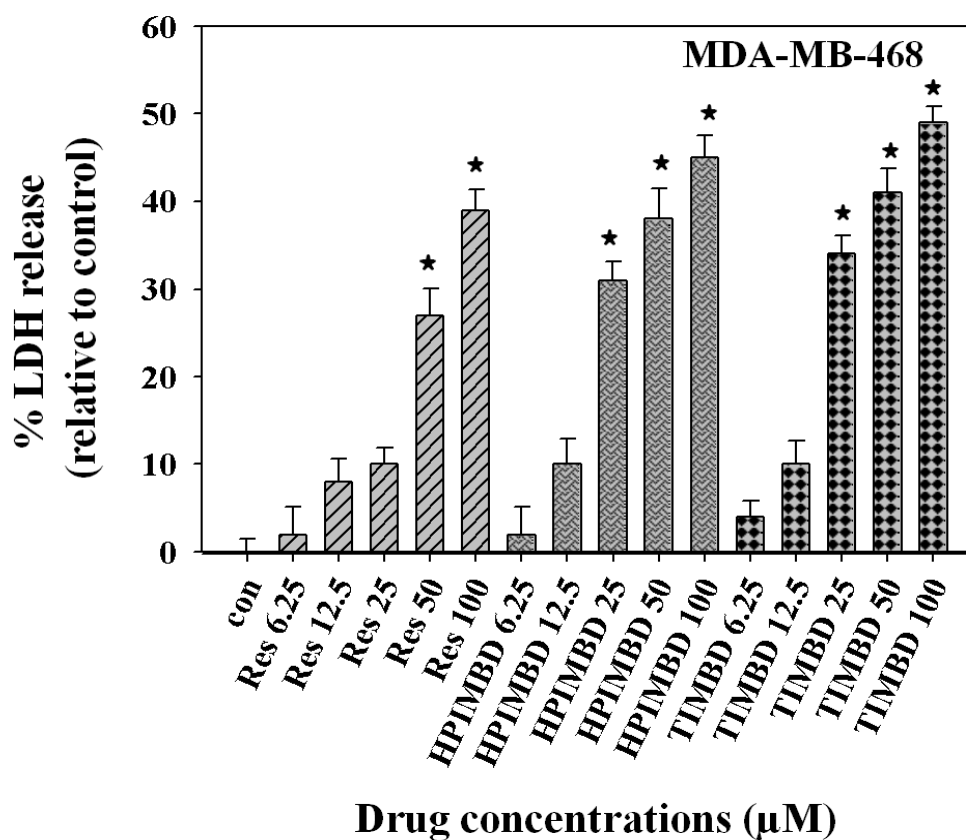
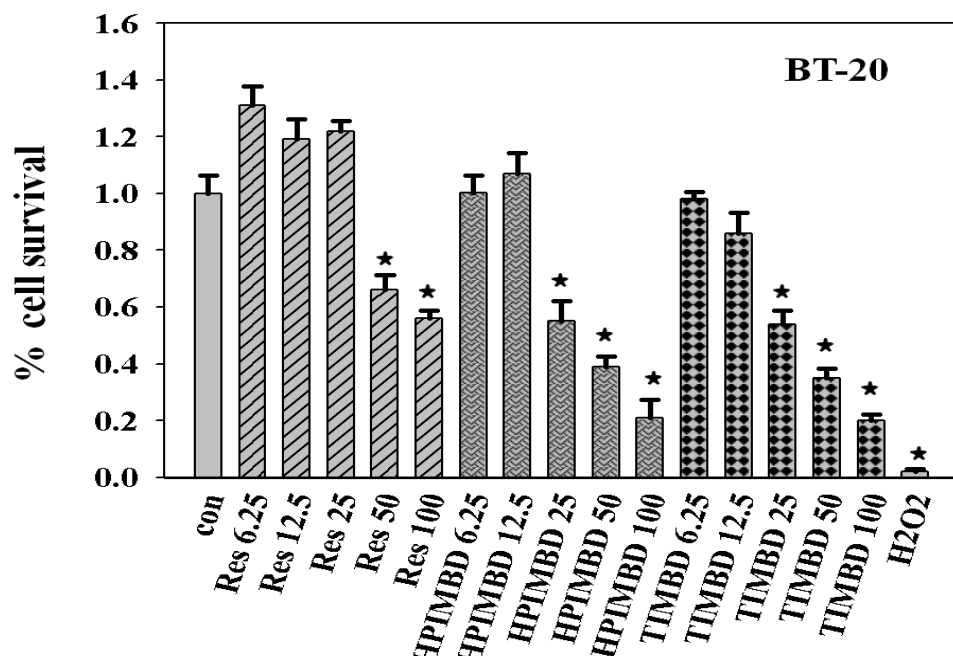**Figure 17:** Cytotoxic activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human triple negative breast cancer cells, MDA-MB-468. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by LDH assay.

* P≤ 0.05 compared to control
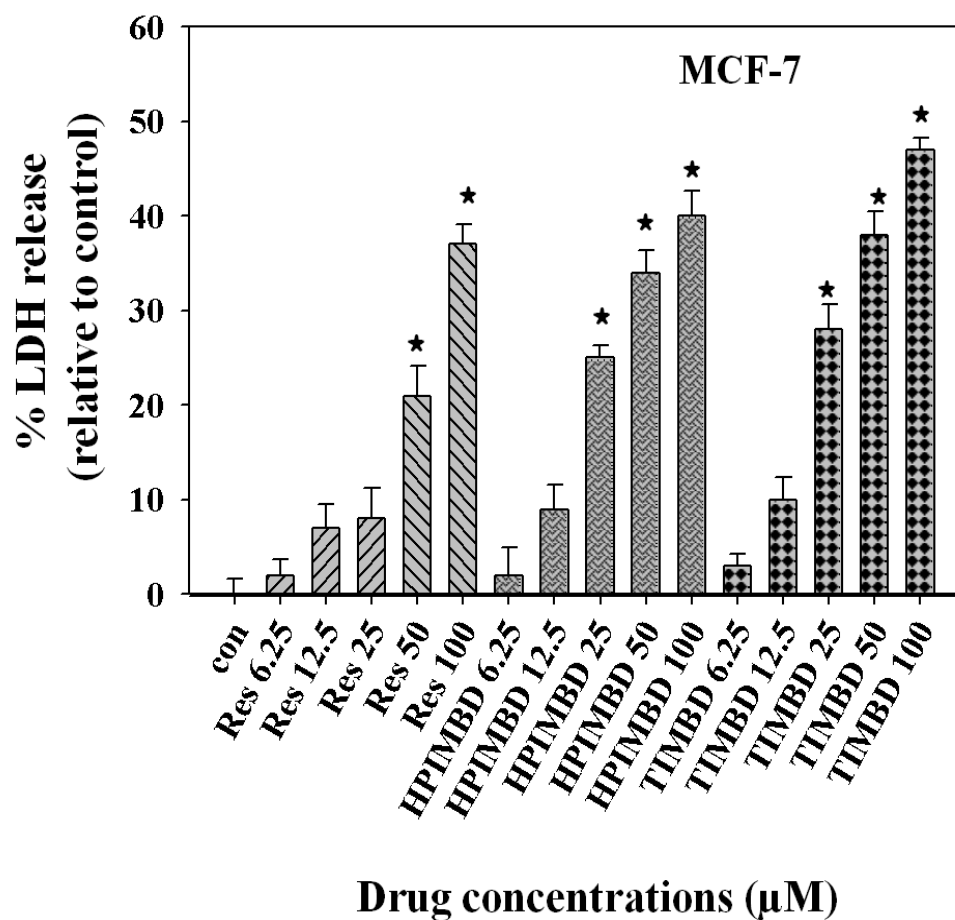
Figure 18: Cytotoxic activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human estrogen-receptor positive breast cancer cells, MCF-7. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by LDH assay.

 * P≤ 0.05 compared to control
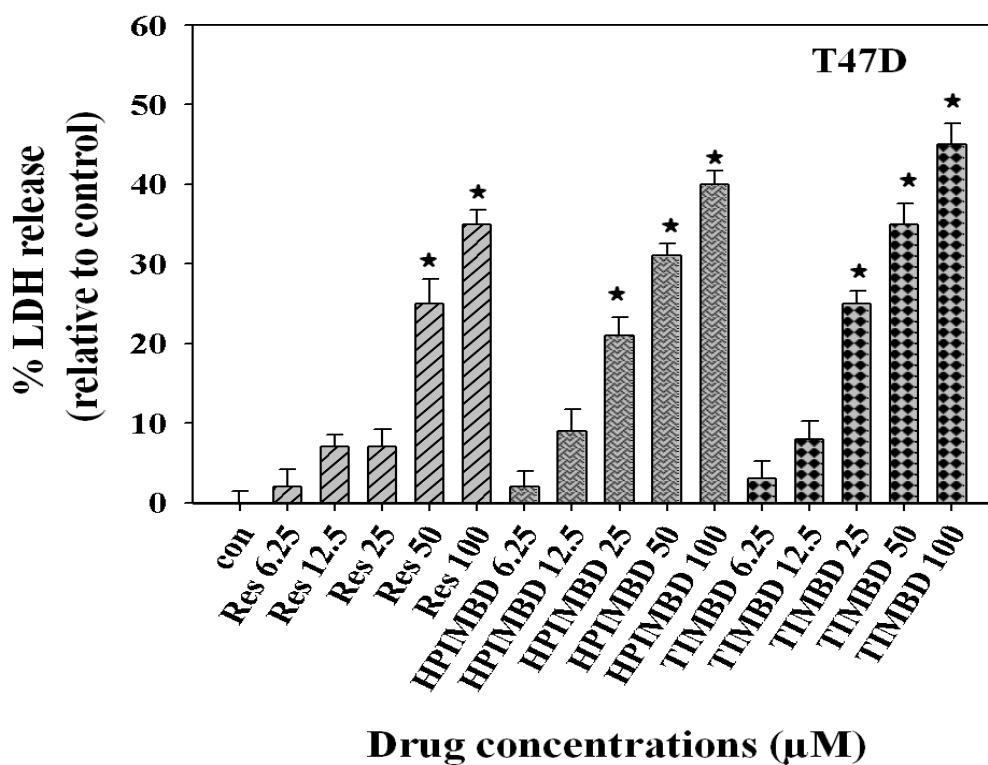
Figure 19: Cytotoxic activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human estrogen-receptor positive breast cancer cells, T47D. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by LDH assay.

 * P≤ 0.05 compared to control
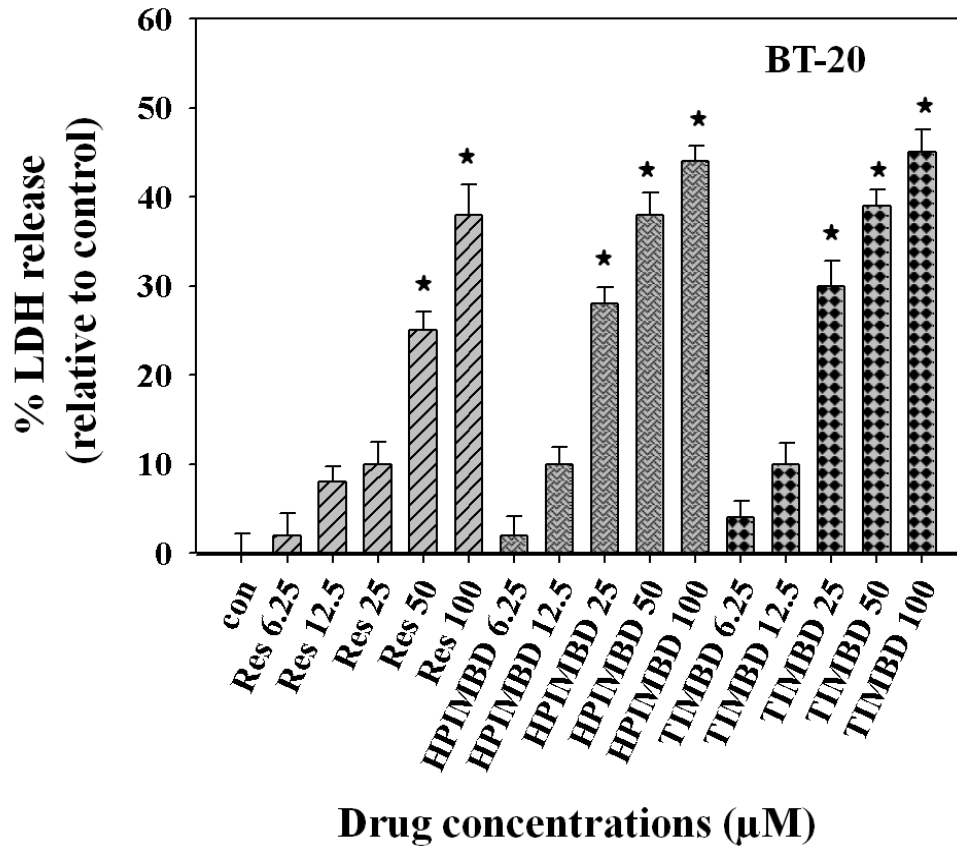
Figure 20: Cytotoxic activity of Aza-resveratrol analogs, HPIMBD and TIMBD against human triple negative breast cancer cells, BT-20. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by LDH assay.
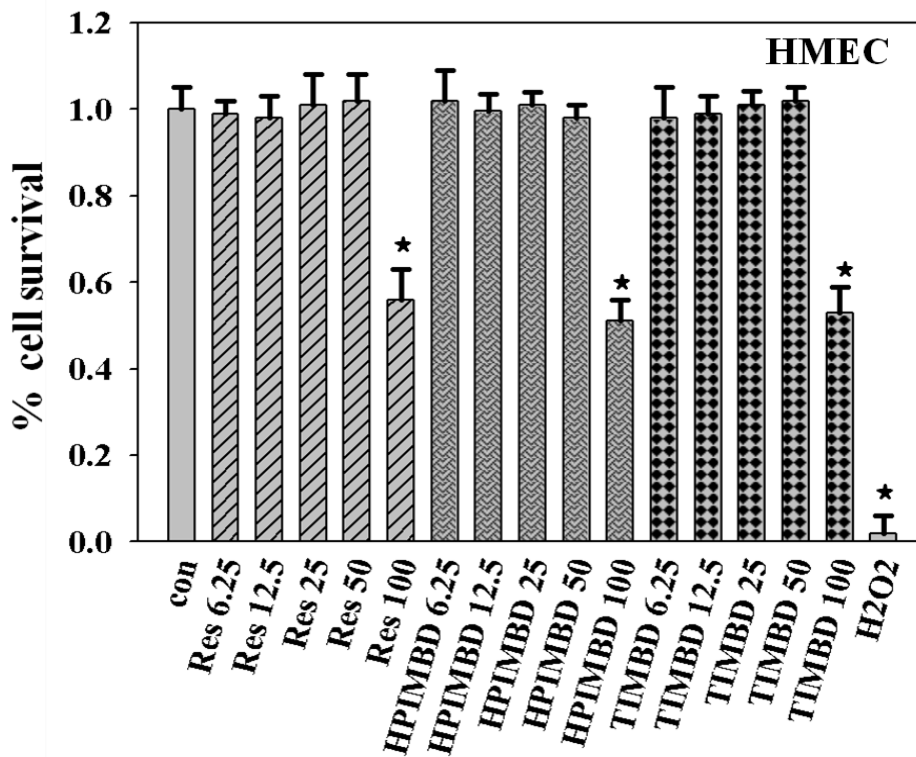
* P≤ 0.05 compared to control
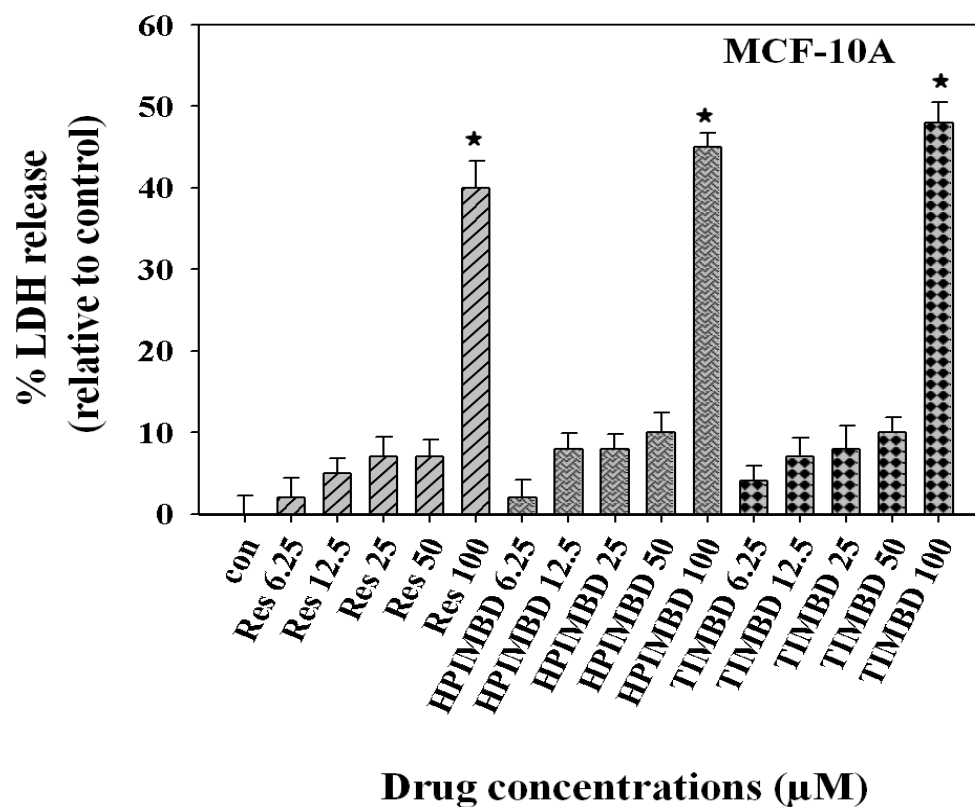
Figure 21: Cytotoxic activity of Aza-resveratrol analogs, HPIMBD and TIMBD against MCF-10A, non-neoplastic human breast epithelial cells. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by LDH assay.

* P≤ 0.05 compared to control
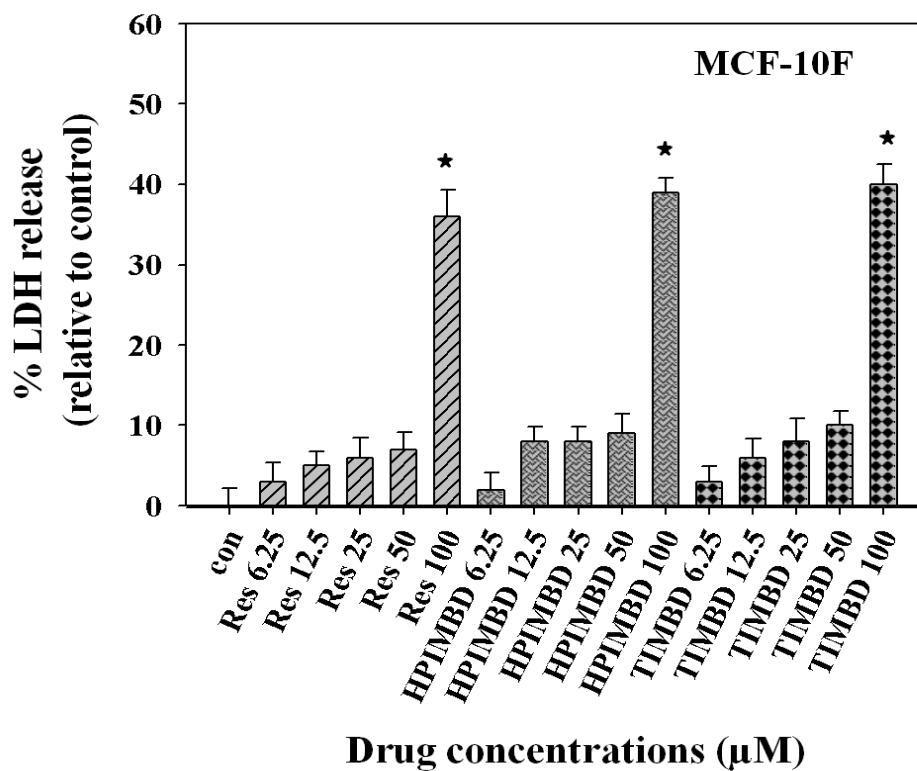
Figure 22: Cytotoxic activity of Aza-resveratrol analogs, HPIMBD and TIMBD against MCF-10F, non-neoplastic human breast epithelial cells. The cells were treated with increasing concentrations of resveratrol (Res), HPIMBD or TIMBD for upto 72 hour and were assessed for cytotoxicity by LDH assay.

* P≤ 0.05 compared to control

3.2.3. Res-analogs are more potent to mediate antioxidant defense by reducing ROS production.

We examined whether treatment with HPIMBD or TIMBD would decrease cellular reactive oxygen species (ROS) levels in MCF-10A cells better than Res. As shown in Figure 23, ROS production was significantly lower when treated with 50 µM of HPIMBD (Figure 23 A) or TIMBD (Figure 23 B) for 6 hour, as compared to control as well as E2. Moreover, treatment with 50 µM of Res did not decrease ROS production in MCF-10A cells. 1 mM concentration of H_2O_2 treatment was used as a positive control (Figure 23 A & B). These observations demonstrate that Res-analogs are more potent to mediate antioxidant defense to the breast epithelial cells by reducing cellular ROS production, and are better than Res.

(A)

(B)
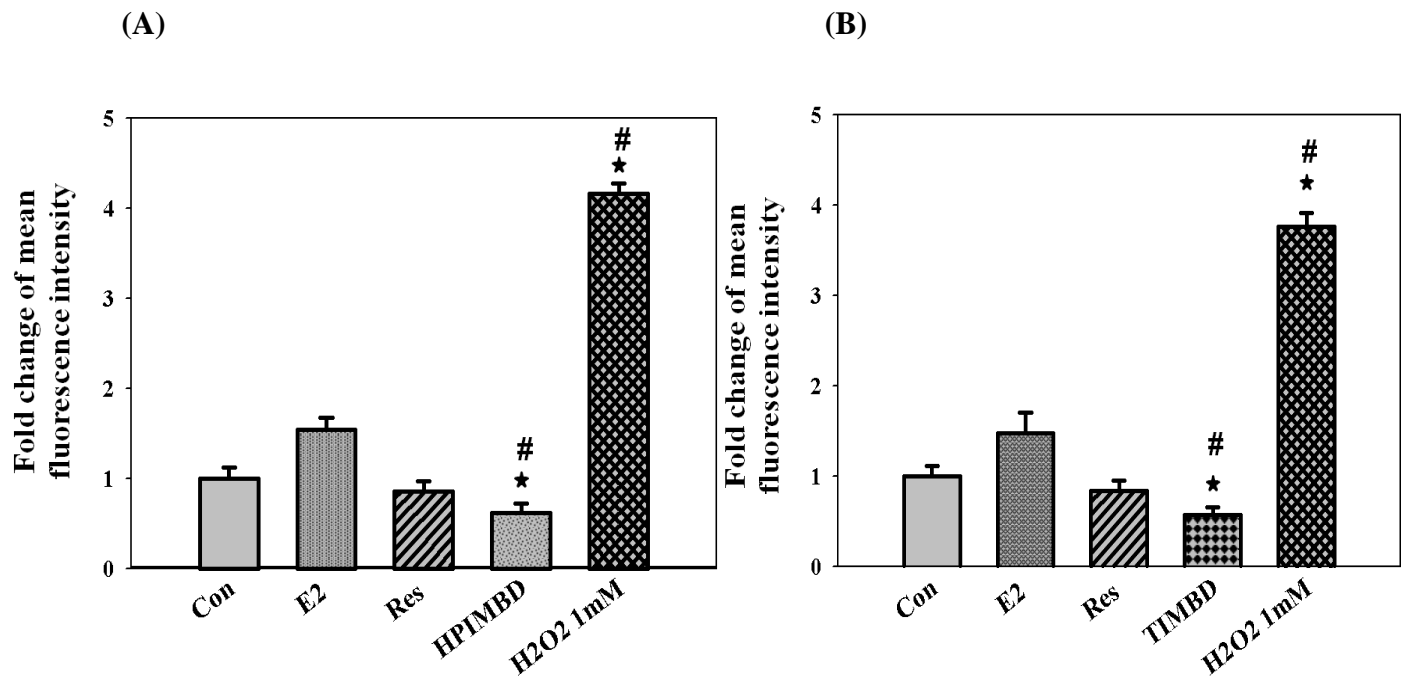
Figure 23: Res-analogs HPIMBD (A) and TIMBD (B) significantly scavenge reactive oxygen species (ROS) in MCF-10A cells treated for 6 hour. * P≤ 0.05 compared to control;   # P≤ 0.05 compared to E2.

3.2.4. Res-analogs are more potent to mediate antioxidant defense by reducing oxidative DNA damage.

DNA 8-OHdG, marker of oxidative DNA damage, was quantified in human non-tumorigenic breast epithelial cells MCF-10A after treatment with Res, TIMBD or HPIMBD in the presence or absence of E2. About 3-fold increase ($P < 0.05$) in 8-OHdG levels was observed in E2-treated MCF-10A cells compared to vehicle treated control (Figure 24 A & B). 8-OHdG levels were significantly decreased in HPIMBD (Figure 24 A) and TIMBD (Figure 24 B)-treated groups as compared to vehicle-treated control in MCF-10A cells. No significant differences in 8-OHdG levels were detected in MCF-10A DNA samples treated with Res, Res + E2, TIMBD + E2 and HPIMBD + E2-treated groups compared to vehicle-treated control but 8-OHdG levels were significantly decreased in Res, Res + E2, TIMBD, TIMBD + E2, HPIMBD and HPIMBD + E2-treated groups as compared to E2 treated MCF-10A cells (Figure 24 A & 24 B). These results suggest the antioxidant properties of TIMBD and HPIMBD in prevention of oxidative DNA damage in breast epithelial cells.
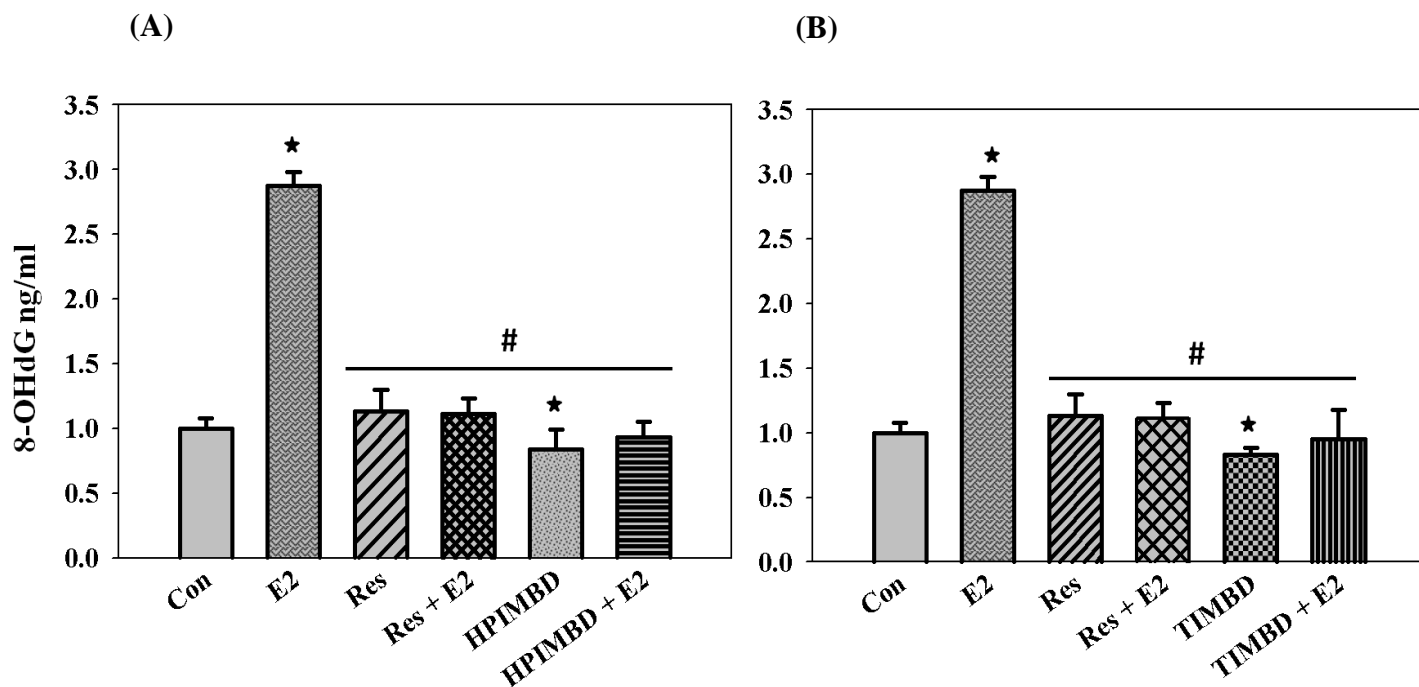
Figure 24: Res-analogs HPIMBD (A) and TIMBD (B) significantly down-regulate oxidative DNA damage in MCF-10A cells treated for 48 hour. * P≤ 0.05 compared to control; # P≤ 0.05 compared to E2.

3.2.5. Natural antioxidants Res and VC reverse estrogen-mediated decrease in NQO1 and SOD3 mRNA and protein expression levels

mRNA and Protein expression levels of NAD(P)H:quinone oxidoreductase 1 (NQO1) were quantified in MCF-10A cells treated with E2 (50 nM) for up to 48 hour. Significant decreases in NQO1 mRNA as well as protein expression levels were determined (Figure 25 A & 25 B). Resveratrol (50 μM) and (1 mM) treatment reversed E2-mediated decrease in NQO1 mRNA as well as protein expression levels and significantly increased NQO1 mRNA and protein expression levels in MCF-10A cells treated for upto 48 hour (Figure 25 A & 25 B). The fold changes in NQO1 protein expression levels in MCF-10A cells treated with E2, Res, Res + E2, VC and VC + E2 for 48 hour were 0.48, 3.28, 3.15, 2.24 and 1.12, respectively, compared to vehicle-treated cells (Figure 25 B). mRNA and protein expression levels of SOD3 were quantified in MCF-10A cells treated with E2 (50 nM) for up to 48 hour. A significant decrease in SOD3 mRNA as well as protein expression levels was detected compared to control cells (Figure 25 C & D). Resveratrol (50 μM) and VC (1 mM) treatment reversed E2-mediated decrease in SOD3 mRNA as well as protein expression levels and further, significantly increased SOD3 mRNA and protein expression levels in MCF-10A cells treated for up to 48 hour (Figure 25 C & 25 D). The fold changes in SOD3 protein expression levels in MCF-10A cells treated with E2, Res, Res + E2, VC and VC + E2 for 48 hour were 0.42, 3.84, 3.79, 2.32 and 2.18, respectively, compared to vehicle-treated controls (Figure 25 D).
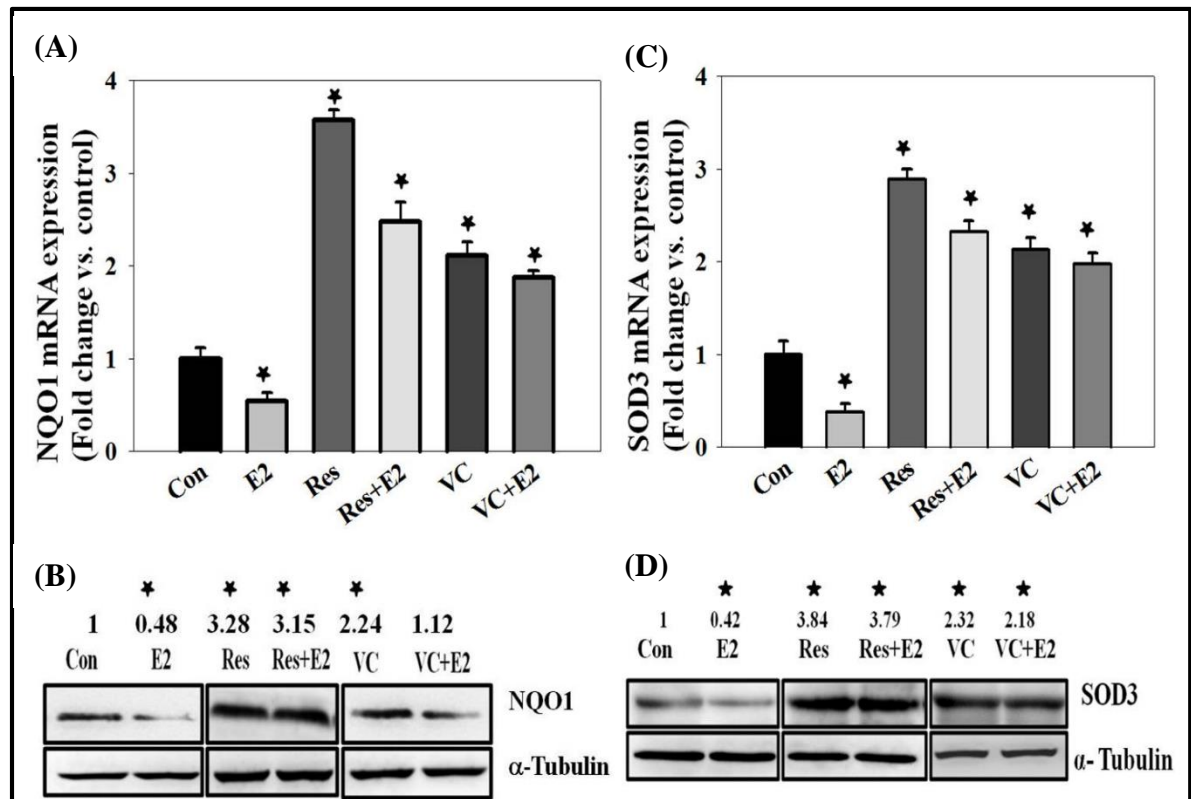
Figure 25: Antioxidants Res and VC reverse E2-mediated decrease in Phase-II detoxifying enzymes, NQO1 and SOD3, at both mRNA and protein levels. (A) NQO1 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res, Res + E2, 1 mM VC or VC + E2 for 24 hour; (B) NQO1 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res, Res + E2, 1mM VC or VC + E2 for 48 hour; (C) SOD3 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res, Res + E2, 1 mM VC or VC + E2 for 24 hour; and (D) SOD3 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res, Res + E2, 1 mM VC or VC + E2 for 48 hour.

'*' indicates *p* value ≤ 0.05 compared with respective controls.

3.2.6. Res-analogs are more potent to mediate antioxidant defense by inducing phase-II detoxifying enzymes SOD3 (specific one from superoxide dismutase family) and NQO1

We wanted to delineate the mechanism of antioxidant defense by Res-analogs TIMBD and HPIMBD. We were interested in finding out if TIMBD and HPIMBD could moderate the cellular levels of phase-II detoxifying enzymes, SOD3 and NQO1. mRNA and protein expression levels of SOD3 were quantified in MCF-10A cells treated with E2 (50 nM) for upto 72 hour. A significant decrease in SOD3 mRNA as well as protein expression levels was detected compared to control cells (Figure 26 & Figure 27). 50 μM of Res or TIMBD treatment reversed E2-mediated decrease in SOD3 mRNA (Figure 26 A) as well as protein (Figure 26 B & C) expression levels and further, significantly increased SOD3 mRNA and protein expression levels in MCF-10A cells treated for upto 72 hour. The fold changes in SOD3 protein expression levels in MCF-10A cells treated with Res or TIMBD for 48 hour were 3.53 and 4.89, respectively, compared to vehicle-treated controls (Figure 26 C). It was also demonstrated that, 50 μM of Res or HPIMBD treatment reversed E2-mediated decrease in SOD3 mRNA (Figure 27 A) as well as protein (Figure 27 B & C) expression levels and further, significantly increased SOD3 mRNA and protein expression levels in MCF-10A cells treated for upto 72 hour. The fold changes in SOD3 protein expression levels in MCF-10A cells treated with Res or HPIMBD for 48 hour were 3.50 and 3.44, respectively, compared to vehicle-treated controls (Figure 27 C).

An enzyme responsible for converting E2-quinones to catechol is NQO1. Our results demonstrated that treatment with 50 μM TIMBD (Figure 28) for upto 72 hour inhibited E2-mediated decrease in NQO1 mRNA (Figure 28 A) and protein (Figure 28 B & C) expression levels and significantly increased NQO1 mRNA protein expression levels in MCF-10A cells. The fold changes in NQO1 protein expression levels in MCF-10A cells treated with Res or TIMBD for 72 hour were 1.25 and 4.23, respectively, compared to vehicle-treated controls (Figure 28 C). It was also demonstrated that treatment with 50 μM HPIMBD (Figure 29) for upto 72 hour inhibited E2-mediated decrease in NQO1 mRNA (Figure 29 A) and protein (Figure 29 B & C) expression levels and further increased NQO1 mRNA and protein expression levels in MCF-10A cells (Figure 29 A, B & C). The fold changes in NQO1 protein expression levels in MCF-10A cells treated with Res or HPIMBD for 72 hour were 1.25 and 4.12, respectively, compared to vehicle-treated controls (Figure 29 C).
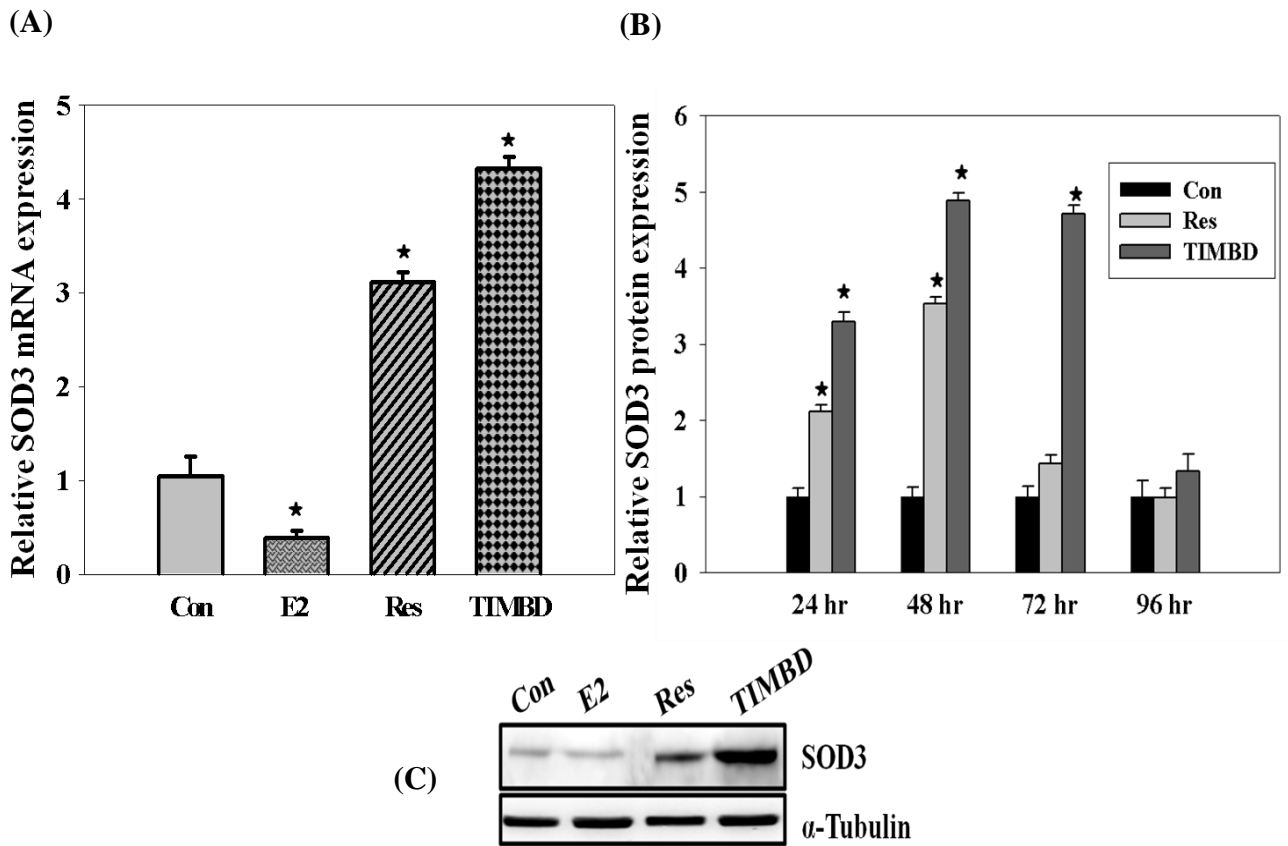
Figure 26: Novel Res-analog TIMBD reverses E2-mediated decrease in Phase-II detoxifying enzyme SOD3, at both mRNA and protein levels. (A) SOD3 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for 24 hour; (B) SOD3 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for upto 96 hour; and (C) Representative Western blot showing increased SOD3 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for 48 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.
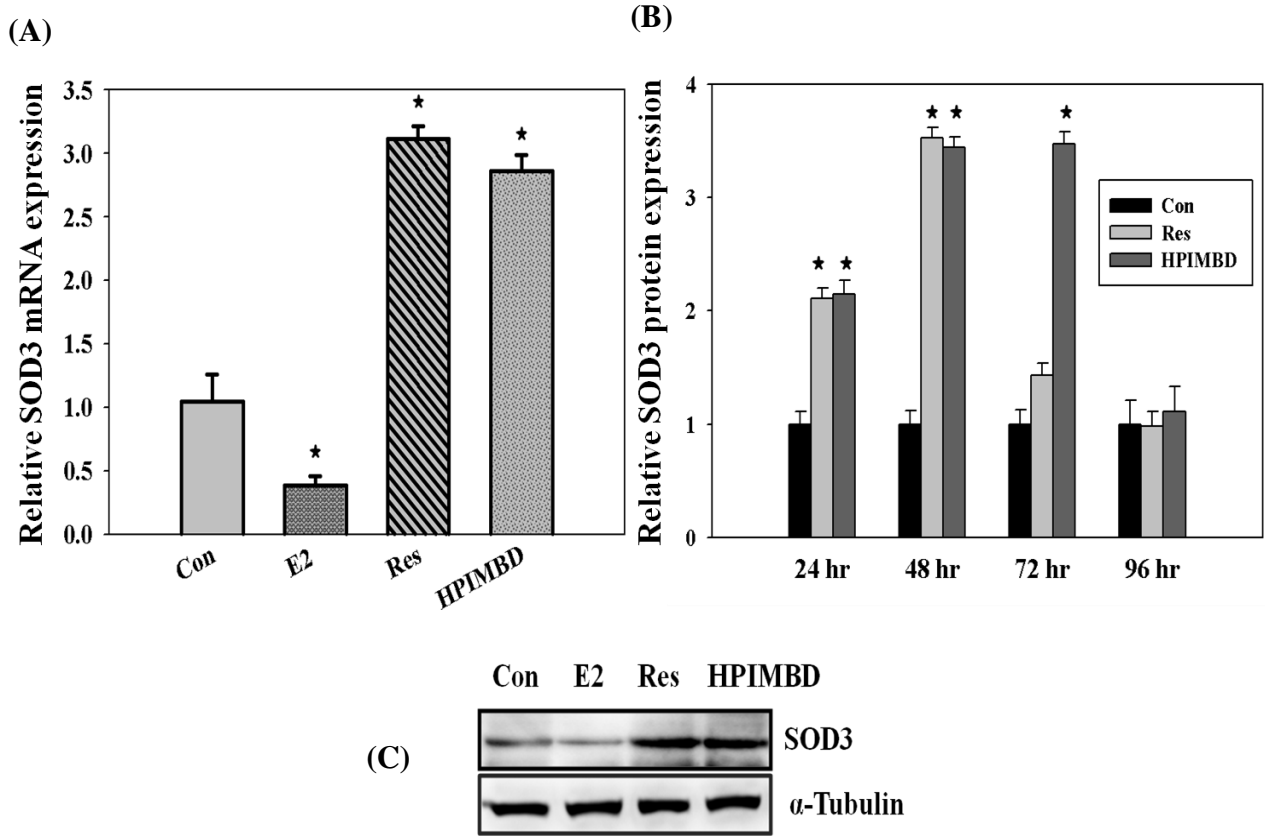
Figure 27: Novel Res-analog HPIMBD reverses E2-mediated decrease in Phase-II detoxifying enzyme SOD3, at both mRNA and protein levels. (A) SOD3 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for 24 hour; (B) SOD3 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for upto 96 hour; and (C) Representative Western blot showing increased SOD3 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for 48 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.
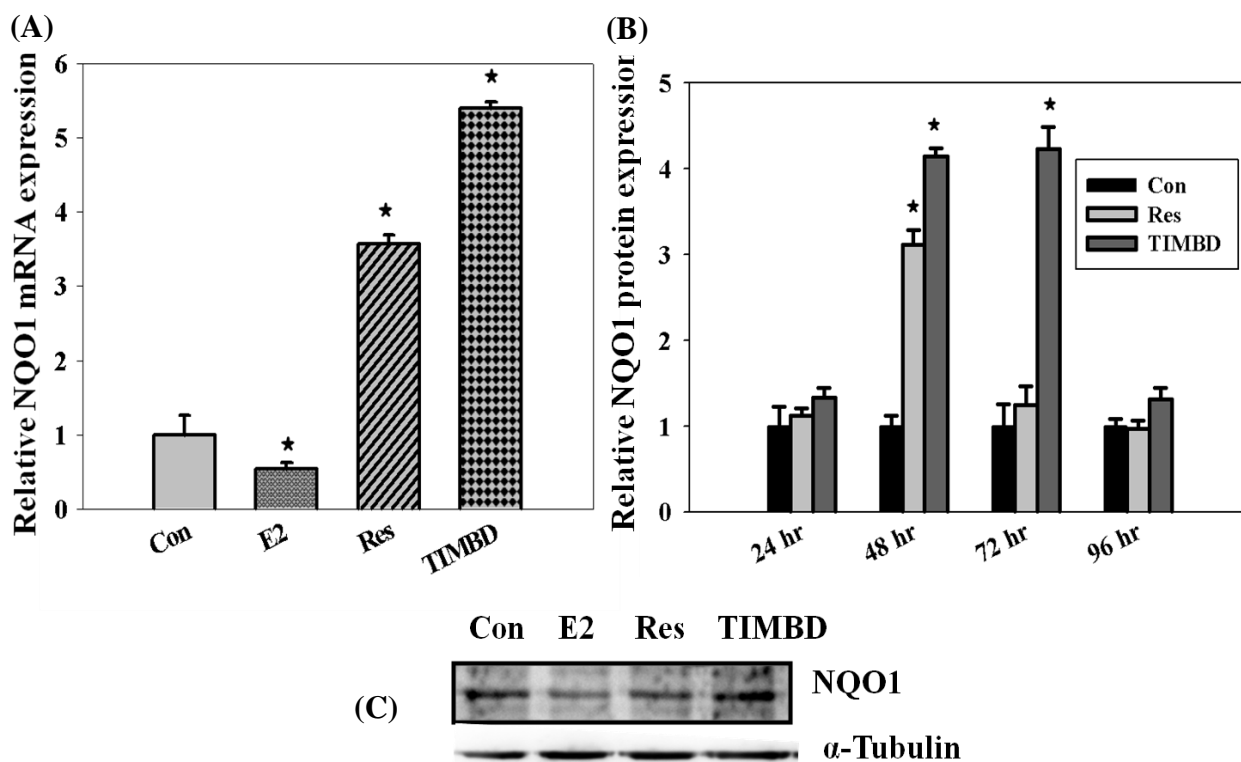
Figure 28: Novel Res-analog TIMBD reverses E2-mediated decrease in Phase-II detoxifying enzyme NQO1, at both mRNA and protein levels. (A) NQO1 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for 24 hour; (B) NQO1 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for upto 96 hour; and (C) Representative Western blot showing increased NQO1 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for 72 hour.

'*' indicates p value ≤ 0.05 compared with respective control

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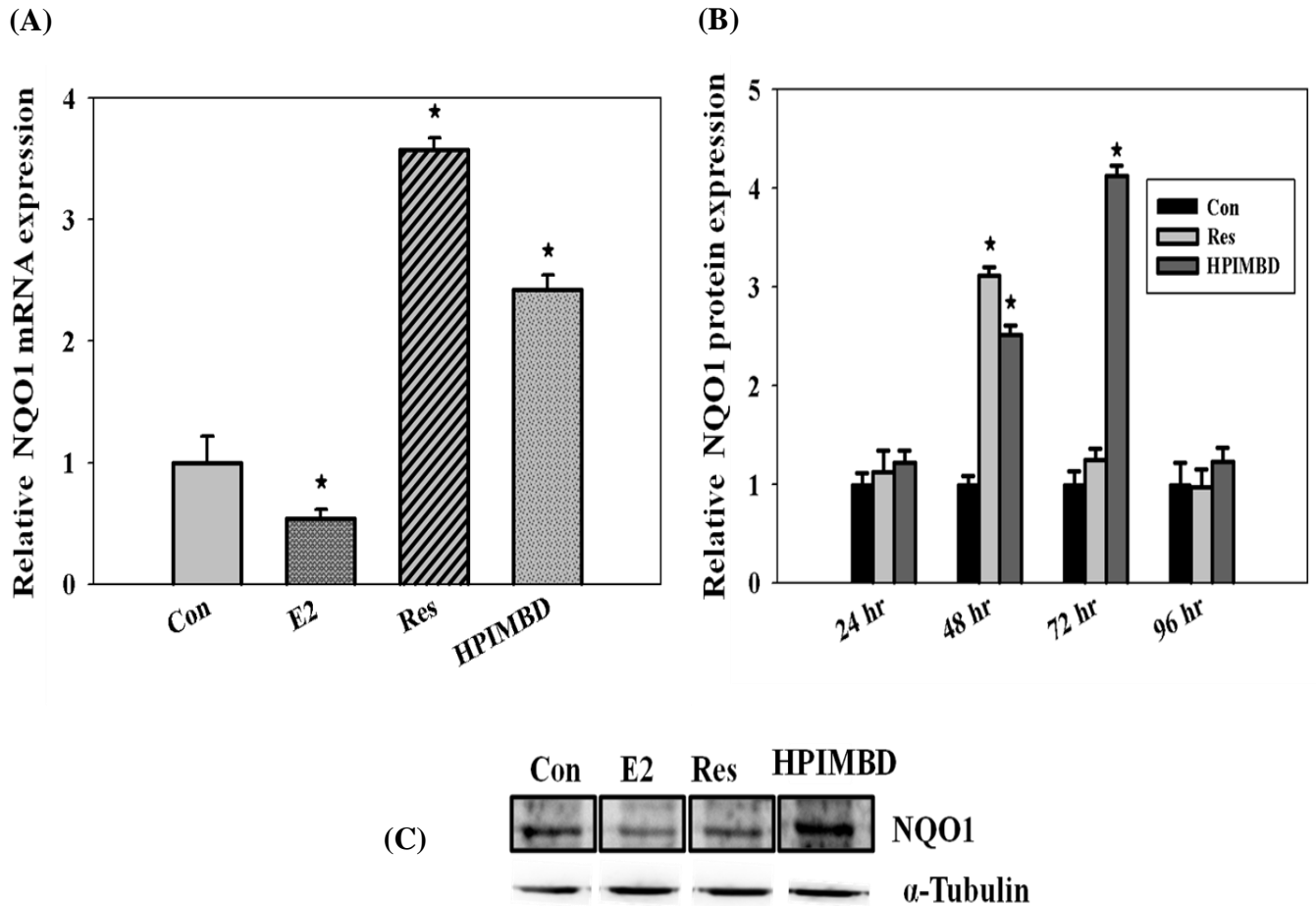
Figure 29: Novel Res-analog HPIMBD reverses E2-mediated decrease in Phase-II detoxifying enzyme NQO1, at both mRNA and protein levels. (A) NQO1 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 µM Res or 50 µM HPIMBD for 24 hour; (B) NQO1 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 µM Res or 50 µM HPIMBD for upto 96 hour; and (C) Representative Western blot showing increased NQO1 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 µM Res or 50 µM HPIMBD for 72 hour.

'*' indicates p value ≤ 0.05 compared with respective control

3.3. Discussion

Long-term exposure to the female sex hormone estrogen has been associated with the initiation and progression of breast cancer (5-7). However, the mechanisms of E2-induced breast carcinogenesis are not clear. It has been suggested that estrogen metabolism plays a major role in the onset of estrogen-induced breast cancer (15). Oxidative stress produced by redox cycling between catechol estrogens and estrogen quinones has been suggested to play a critical role in estrogen-induced breast cancer (5, 6). These reactive oxygen species generated during estrogen-redox cycling can react with DNA to form apurinating adducts that are more likely to result in DNA mutations and ultimately causing cancer (37).

In spite of all these studies suggesting important role of oxidative stress in development of breast cancer, the underlying mechanism is still not clearly understood. Therefore, it is of utmost importance to dissect out the molecular mechanisms of oxidative stress mediated breast cancer, of particular E2-mediated breast cancer and, accordingly, develop therapeutic interventions that target these pathways.

A plenty of non-synthetic natural compounds are found with unique medicinal values. One of the hugely studied naturally occurring plant derived phyto-estrogen is namely resveratrol (Res), well studied for its medicinal properties. It is found in various vegetables, nuts, fruits including grapes and red wine. Resveratrol has been well explored for its plethora of medicinal properties in treatment of cancer and a wide range of other diseases (89-94). Resveratrol mediates its anticancer activity by inducing apoptosis in cancer cells via modulating various signal transduction pathways and targeting different transcription factors responsible for uncontrolled growth of cancer cells (115-119).

Although Res has been successfully shown to reduce growth of primary tumor xenografts in rodent (120), its use has been limited in clinical applications due to its poor effectiveness in human body (121-125). Therefore, pharmacologically active Res-analogs have been synthesized by our collaborative team (133) in order to improve its antioxidant and anticancer efficacy. The analogs synthesized were named as HPIMBD and TIMBD (133), have extensively studied for their antiproliferative properties on breast cancer cells. TIMBD and HPIMBD have shown to inhibit breast cancer cell proliferation effectively and successfully with no or least toxicity towards normal breast epithelial cells.

In this current work we have shown the potency of TIMBD and HPIMBD in preferentially inhibiting the proliferation of breast cancer cells. We found the IC_{50} of TIMBD and HPIMBD is <5 µM in the breast cancer cell line MDA-MB-468 derived from African American breast cancer patient. It is reported that IC_{50} value of <5 µM is well within the acceptable range for evaluation of a drug for efficacy and safety in a clinical setting (139, 140). Our novel compounds TIMBD and HPIMBD were tested for their antiproliferative and cytotoxic effects against human breast cancer cell lines MCF-7, T47D, MDA-MB-231, MDA-MB-468, BT-20 as well as human normal breast cell lines MCF-10A, MCF-10F and HMEC by MTT and LDH assays, respectively. These compounds were compared with Res for their cytotoxic effects on above-mentioned cell lines. Our novel compounds TIMBD and HPIMBD exhibited antiproliferative effects (Figures 8-12) and cytotoxic effects (Figures 16-20) against all breast cancer cell lines and they showed better cytotoxicity than Res. The compounds TIMBD and HPIMBD exhibited IC50 value of 21 and 29 µM against MDA-MB-231, and 32 and 44 µM against

T47D breast cancer cell lines, respectively, as compared to Res with IC_{50} value of 66 and 76 μM, respectively (Table 2). None of the novel-analogs exhibited any antiproliferative (Figures 13-15) or cytotoxic (Figures 21 & Figure 22) effects against human non-tumorigenic breast epithelial cells MCF-10A, MCF-10F and HMEC at 50 μM concentrations, which suggest the safety of our novel Res-analogs towards normal breast epithelial cells and specificity towards cancer cells. Thus, in this current study we aimed at developing TIMBD and HPIMBD as a novel therapeutic compound for treatment of breast cancer with minimal or no toxicity towards normal breast epithelial cells.

Studies from our laboratory and that of others have already shown oxidative-DNA damage induced by estrogen (36, 37). In this present study we wanted to examine the protective effects of Res-analogs TIMBD, HPIMBD as well as prototypic antioxidant Res in inhibition of cellular ROS production and inhibition of oxidative DNA damage in MCF-10A cells. We measured the levels of ROS in MCF-10A cells treated with 50 nM E2, 50 μM Res, 50 μM TIMBD or 50 μM HPIMBD for 6 hour (Figure 23). We demonstrated that treatment with TIMBD or HPIMBD significantly decreased ROS production in MCF-10A cells as compared to E2 as well as vehicle-treated control (Figure 23). 50 μM Res did not decrease ROS production in MCF-10A cells treated for 6 hour. These observations demonstrate that Res-analogs are more potent to mediate antioxidant defense to the breast epithelial cells by reducing cellular ROS production, and are better than Res. We measured the levels of 8-OHdG which is a known marker of oxidative DNA damage in MCF-10A cells treated with Res, TIMBD or HPIMBD with or without E2 for 48 hour. We demonstrated that E2-treatment significantly increased 8-OHdG levels in MCF-10A cells (Figure 24 A & 24 B). 8-OHdG levels were significantly

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decreased in HPIMBD (Figure 24 A) and TIMBD (Figure 24 B)-treated cells as compared to vehicle-treated control, but not with Res-treatment (Figure 24 A & 24 B). These studies suggest a protective role of TIMBD and HPIMBD against oxidative DNA damage (Figure 24 A & 24 B).

In order to elucidate the molecular mechanisms by which Res-analogs TIMBD and HPIMBD mediate antioxidant defense to the human breast-epithelial cells, we targeted to study their effects in regulation of phase-II detoxifying enzymes. A large number of epidemiological, clinical and experimental evidences provide newer approaches for cancer chemoprevention (141-144). Induction of antioxidant phase-II detoxifying enzyme(s) is one such approach for breast cancer chemoprevention that can be used to inhibit the overproduction of oxidative stress in the process of estrogen induced breast cancer (36, 49, 145, 146). The phase II detoxifying enzymes such as SOD and NQO1 are known to scavenge harmful reactive oxygen species. The role of Superoxide dismutase 3 (SOD3) in prevention of E2-induced breast cancer has been reported recently (49). SOD3 is a key enzyme involved in regulation of oxidative stress, and its increased expression can prevent estrogen-induced breast cancer. In this respect, we have shown in our previous studies that mRNA, protein and enzyme activity of SOD3 are reduced in E2-induced breast tumors and in E2-exposed breast tissue in experimental animals, and increased by antioxidants (49). In this present study we wanted to delineate the mechanism by which Res-analogs, TIMBD and HPIMBD act as potent antioxidants in breast cancer and examined their effects on the expression levels of SOD3. In this regard, we have shown that mRNA and protein expression levels of SOD3 are reduced in E2-treated MCF-10A cells, and significantly increased by TIMBD (Figure 26 A, B & C),

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HPIMBD (Figure 27 A, B & C) as well as prototypic antioxidants Res and VC (Figure 25 C & 25 D). Furthermore, we have shown that our Res-analogs are better than Res in up-regulation of SOD3 mRNA and protein expression levels (Figure 27 7 Figure 27). NAD(P)H-quinone oxidoreductase 1 (NQO1) is another very important and well studied phase-II detoxifying enzyme that has been recently shown to play important role in E2-induced breast cancer (36). NQO1 is a key enzyme which converts E2-quinones to E2-catechols and thus reduces oxidative stress generated by E2-metabolism and subsequent carcinogenic process. It has been reported that NQO1 plays important role in chemical-induced cancer (147-149). Recent work from our laboratory has shown E2-mediated decrease in the expression of NQO1 mRNA and protein which was reversed by antioxidants or Res in female ACI rats (36), which further supports the importance of antioxidant phase-II detoxifying enzyme NQO1 in prevention of breast cancer. In the present study, we examined the role of TIMBD and HPIMBD in mediating antioxidant defense through regulation of NQO1. We have demonstrated that suppression of NQO1 mRNA and protein expression levels following E2 treatment can be reversed upon co-treatment with Res-analogs TIMBD (Figure 28), HPIMBD (Figure 29) as well as prototypic antioxidants VC or Res (Figure 25 A & 25 B). Furthermore, we have shown that TIMBD-treatment mediated highest induction of NQO1 mRNA and protein expression levels (Figure 28). Collectively, these results demonstrate protective role of Res-analogs, TIMBD and HPIMBD in E2-induced breast cancer as potent antioxidants.

In summary, we have presented evidence that our novel Res-analogs TIMBD and HPIMBD inhibit proliferation in breast cancer cells better than resveratrol and have no effect on non-tumorigenic breast epithelial cells. These Res-analogs are more potent to

mediate antioxidant defense by reducing ROS production, oxidative DNA damage and inducing phase-II detoxifying enzymes SOD3 (specific one from superoxide dismutase family) and NQO1 in E2-induced breast cancer and may play important roles in the regulation of cellular adaptive antioxidant responses against E2-induced breast cancer.

CHAPTER 4

DETERMINATION OF THE MECHANISM OF INHIBITION OF E2-INDUCED
BREAST CANCER BY RES-ANALOGS THROUGH REGULATION OF CNC-B ZIP
TRANSCRIPTION FACTORS (NRFS)

4.1. Introduction

Transcription factors from Cap'n'collar (CNC) family of proteins are well known among vertebrates for mediating important developmental and homeostatic functions (150-153). The vertebrate nuclear factor erythroid 2-related factor 1 (Nrf1, also called NFE2L1), nuclear factor erythroid 2-related factor 2 (Nrf2), and nuclear factor erythroid 2-related factor 3 (Nrf3) comprise a subgroup of CNC factors that mediate adaptive responses to cellular stresses (150-153). The most studied stress-activated CNC factor is Nrf2, which is responsible for the transcriptional response of cells to oxidative stress mediators and electrophilic xenobiotics (154-158). The three Nrfs have broad and partly overlapping expression patterns and functions as stress-activated transcription factors. Although, functions of Nrf2 in controlling oxidative stress have been studied, there are fewer studies reporting functions of Nrf1 in controlling oxidative stress and estrogen-induced breast cancer. It has been suggested that Nrf1 may play as important a role in human carcinogenesis as Nrf2 (159-162). The transcription factor Nrf2 is known to be under homeostatic control by interaction with Kelch-like ECH-associated protein 1 (Keap1) which favors its rapid ubiquitination and degradation by the proteasome (154, 155). When the cell encounters oxidative stress, the Keap1-mediated proteasomal

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degradation of Nrf2 is compromised allowing Nrf2 to dissociate from Keap1 and translocate to the nucleus to activate antioxidant gene expressions (158). Studies have shown that homeostatic Keap1-Nrf2 interactions are not permanent and take place in the nucleus via shuttling of Keap1 into that compartment (154, 158). In contrast, Nrf1 is not regulated by Keap1 instead, the activity of Nrf1 appears to be negatively controlled by its N-terminal domain (NTD), which directs Nrf1 to the endoplasmic reticulum (163). Nrf1, but not Nrf2 or Nrf3, is essential for embryonic development; *Nrf1-/-* mice die at mid-late gestation, presumably due to anemia-induced hypoxia (153). In contrast, Nrf3 is known to negatively regulate antioxidant enzymes (164).

Since Nrf1, Nrf2 and Nrf3 control phase II detoxification enzymes that help in detoxification of potential carcinogens, understanding the mechanisms of regulation of these three "Nrfs" may help in the development of cancer therapeutics. Further, due to the cytoprotective nature of phase II enzymes against oxidative stress-induced diseases including cancer, manipulation of the upstream transcription factors controlling these factors (e.g., Nrf1, Nrf2 and Nrf3) or enzymes superoxide dismutases 1, 2, 3 (SOD1, SOD2, SOD3) and NAD(P)H-quinone oxidoreductase 1 (NQO1) could be useful in the search of therapeutic targets against estrogen- induced breast cancer. We report in this study that natural antioxidants Res and and our novel Res-analogs TIMBD and HPIMBD may mediate their protective role in E2-induced breast cancer through a complex interplay of CNC b-zip family of proteins.

4.2 Results

4.2.1. Estrogen decreases, whereas antioxidants reverse E2-mediated decrease in Nrf2 mRNA and protein expression levels, and further increase its mRNA and protein expression levels.

A dose-dependent change in Nrf2 mRNA and protein expression levels was determined using different concentrations of E2. A maximum decrease in Nrf2 mRNA (24 hour) and protein expression (48 hour) at a dose of 50 nM E2 was observed (Figure 30 A & 30 B). A time-dependent change in Nrf2 mRNA and protein expression levels was determined using 50 nM E2. A maximal inhibition of Nrf2 mRNA expression at 24 hour and protein expression at 48 hour was observed (Figure 30 C & 30 D). The effect of Res and VC on the regulation of Nrf2 mRNA and protein expression levels in MCF-10A cells was examined. Vitamin C (1 mM) or Res (50 μM) significantly reversed E2-mediated decrease in Nrf2 mRNA and protein expression levels and further increased Nrf2 mRNA and protein expression levels in MCF-10A cells after 48 hour of treatment (Figure 30 E & 30 F). The fold changes in Nrf2 protein expression levels in MCF-10A cells treated with E2, Res, Res + E2, VC and VC + E2 for 48 hour were 0.43, 2.33, 1.87, 3.32 and 2.84, respectively, compared to vehicle-treated control cells (Figure 30 F).
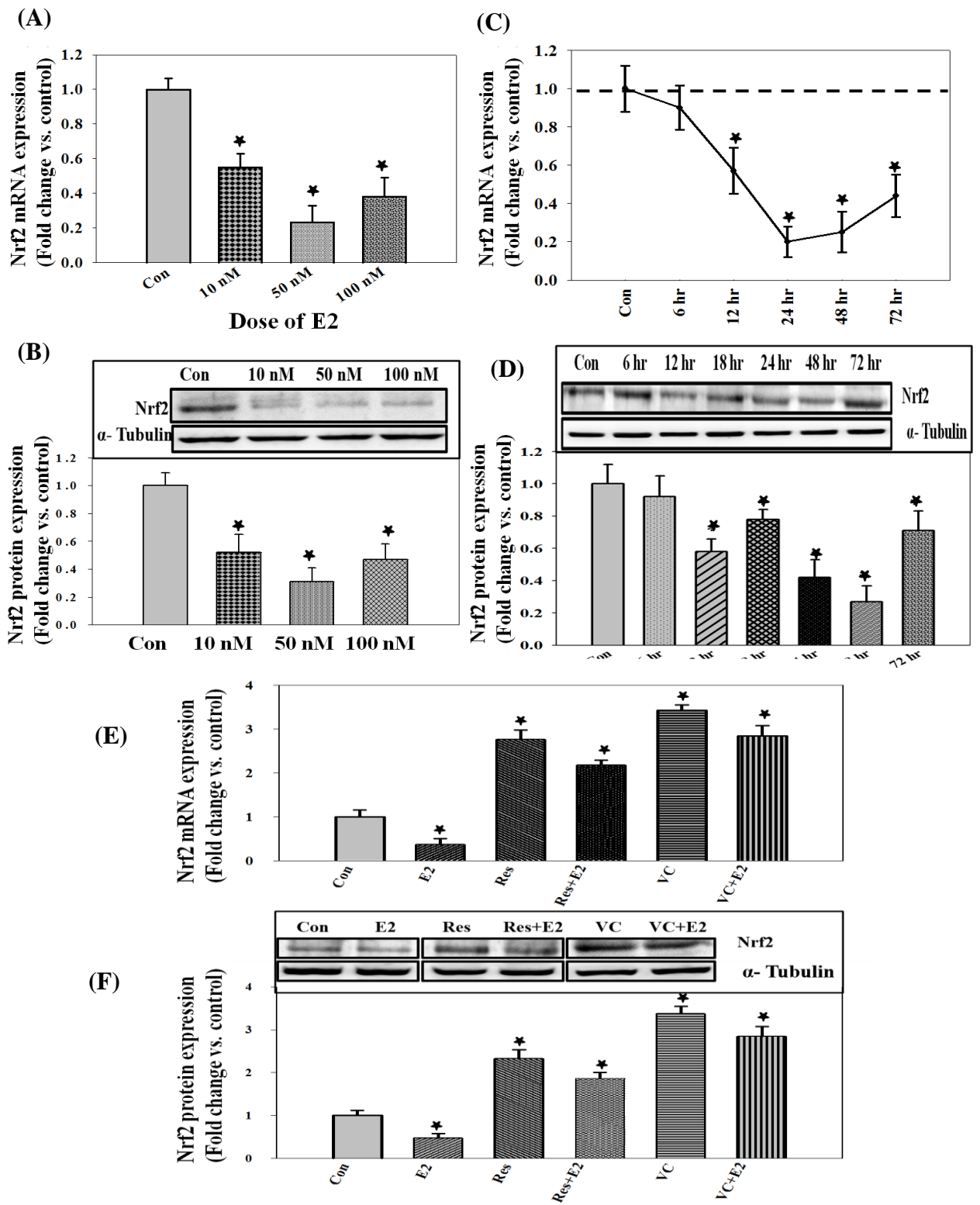
Figure 30: 17 β-Estradiol (E2) down-regulates, whereas antioxidants reverse E2-mediated decrease in Nrf2 mRNA and protein expression levels, and further increase its mRNA and protein expression levels in MCF-10A cells. (A) Nrf2 mRNA expression levels in MCF-10A cells treated with 10, 50 and 100 nM E2 for 24 hour; (B) Nrf2 protein expression levels in MCF-10A cells treated with increased doses of E2 for 48 hour; (C) Nrf2 mRNA expression levels in MCF-10A cell line treated with 50 nM E2 for upto 72 hour; (D) Nrf2 protein expression levels in MCF-10A cell line treated with 50 nM E2 for upto 72 hour; (E) Antioxidants (VC and Res) significantly reverse E2-mediated decrease in Nrf2 mRNA and expression levels in MCF-10A cells; cells were treated with 50 nM E2, 50 μM Res, Res + E2, 1 mM VC or VC + E2 for 24 hour; and (F) Nrf2 protein expression levels in MCF-10A cell line treated with 50 nM E2, 50 μM Res, Res + E2, 1 mM VC or VC + E2 for upto 48 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.

4.2.2. Estrogen treatment induces Nrf3 and Nrf1, whereas antioxidants differentially regulate Nrf3 and Nrf1 in MCF-10A cells.

Nrf3 mRNA and protein expression levels were quantified in MCF-10A cells treated with E2 (50 nM) for upto 72 hour. A significant increase in Nrf3 mRNA as well as protein expression levels compared to control was identified at 48 hour after treatment (Figure 31 A & 31 B). Resveratrol (50 µM) and VC (1 mM) treatment reversed E2-mediated increase in Nrf3 mRNA as well as protein expression levels, and significantly decreased Nrf3 mRNA and protein expression levels in MCF-10A cells compared to controls (Figure 31 A & 31 B). The fold changes in Nrf3 protein expression levels in MCF-10A cells treated with E2, Res, Res + E2, VC and VC + E2 for 48 hour were 2.68, 0.53, 0.31, 0.61 and 0.78, respectively, compared to vehicle-treated control cells (Figure 31 B). Nrf1 mRNA and protein expression levels were quantified in MCF-10A cells treated with E2 (50 nM) for upto 48 hour. A significant increase in Nrf1 mRNA as well as protein expression levels compared to control was detected (Figure 31 C & 31 D). Antioxidants by themselves also significantly increased Nrf1 mRNA and protein expression levels compared to vehicle-treated control cells (Figure 31 C & 31 D). This increase was independent of E2 treatment. The fold change in Nrf1 protein expression levels in MCF-10A cells treated with E2, Res, Res + E2, VC and VC + E2 for upto 48 hour were 2.87, 2.51, 2.62, 2.13 and 1.97, respectively, compared to vehicle-treated control cells (Figure 31 D).

(A)

Nrf3 mRNA expression (Fold change vs. control)

Legend: Con, E2, Res, Res+E2, VC, VC+E2

x-axis: 12 hr, 24 hr, 48 hr, 72 hr

(B)

Con E2 Res Res+E2 VC VC+E2

Nrf3

α-Tubulin

Nrf3 protein expression (Fold change vs. control)

x-axis: Con, E2, Res, Res+E2, VC, VC+E2

(C)

Nrf1 mRNA expression (Fold change vs. control)

Legend: Con, E2, Res, Res+E2, VC, VC+E2

x-axis: 12 hr, 24 hr, 48 hr

(D)

Con E2 VC VC+E2 Res Res+E2

Nrf1

α-Tubulin

Nrf1 protein expression (Fold change vs. control)

x-axis: Con, E2, VC, VC+E2, Res, Res+E2

Figure 31: 17 β-Estradiol induces Nrf3 as well as Nrf1 mRNA and protein expression levels in MCF-10A cells. Antioxidants (VC and Res) reverse E2-mediated increase in Nrf3 mRNA and protein expression levels and induce Nrf1 mRNA and protein expression levels in MCF-10A cells. (A) Nrf3 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res, Res + E2, 1 mM VC or VC + E2 for upto 72 hour; (B) Nrf3 protein expression levels in MCF-10A cells treated with 50 nM E2 and 50 μM Res, Res + E2, 1 mM VC or VC + E2 for 48 hour; (C) Nrf1 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res, Res + E2, 1 mM VC or VC + E2 for upto 48 hour; and (D) Nrf1 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res, Res + E2, 1 mM VC or VC + E2 for 48 hour.

4.2.3. Nrf3 negatively regulates Nrf2 and Nrf2-dependent gene NQO1

Nrf2 is a known positive regulator of antioxidant phase-II detoxifying gene NQO1 (36), whereas Nrf3 is known to negatively regulate NQO1 gene (164). We investigated whether silencing of Nrf3 has any effect on the expression of Nrf2 gene. An increase in Nrf2 protein expression was observed after silencing of Nrf3 in MCF-10A cells (Figure 32). Additionally an increase in NQO1 protein expression after silencing of Nrf3 was detected (Figure 32).
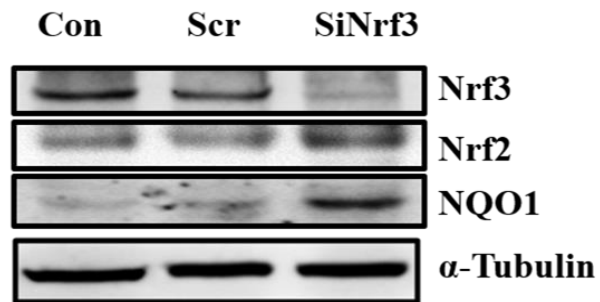
Figure 32: Nrf3 negatively regulates Nrf2 and Nrf2-dependent gene NQO1. MCF-10A cells were transfected with 20 nmol/l of scrambled siRNA or siNrf3 for 48 hour, and Western blot analysis was carried out using Nrf3 antibody. The same membrane was reprobed with Nrf2, NQO1 and α-tubulin antibodies.

4.2.4. Nrf3 interacts with Nrf2.

Co-immunoprecipitation is an *in vitro* method for detecting protein-protein interactions. We used Pierce Co-immunoprecipitation Kit to investigate whether Nrf2 interacts with Nrf3 and *vice versa* as described in the methods section. After immobilization and immunoprecipitation of the primary antibody (Nrf2), Co-Ip of interacting protein Nrf3 was detected by western blotting (Figure 33 i). The same blot was stripped and probed for Nrf2 and a mild band was detected (Figure 33 ii). After immobilization and immunoprecipitation of the primary antibody (Nrf3), Co-Ip of interacting protein Nrf2 was detected by Western blotting (Figure 33 iii). The same blot was stripped and probed for Nrf3 and Nrf3 was detected (Figure 33 iv). Any unspecific interactions were identified by using the provided control gel and substituting IgG for the specific antibody. No nonspecific interaction was observed (Figure 33).
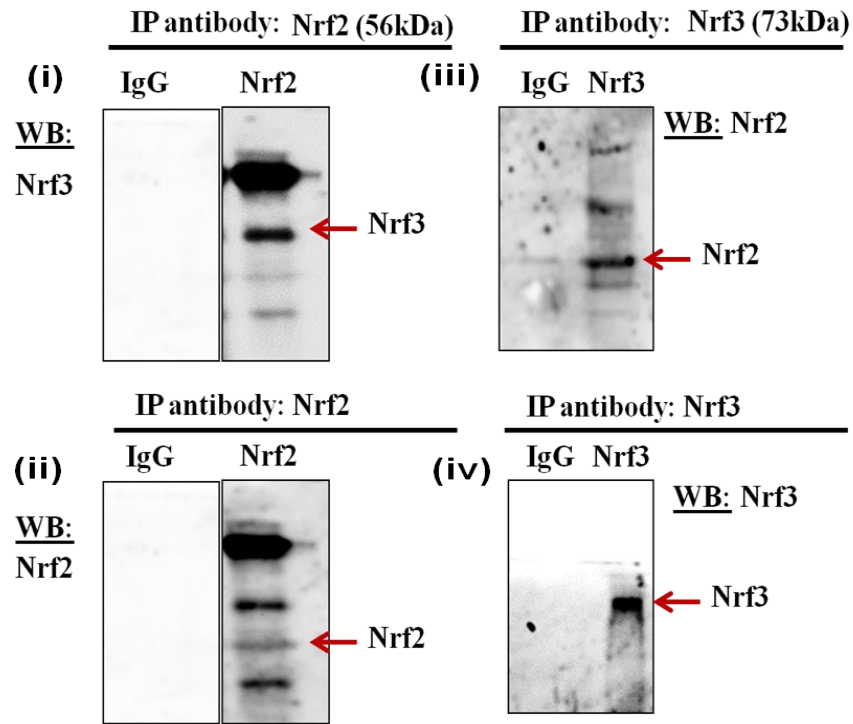
Figure 33: Co-Immunoprecipitation of Nrf2 and Nrf3 from MCF-10A cells. Co-Immunoprecipitation of Nrf2 and Nrf3 were performed in MCF-10A cell lysates using Pierce Co-Ip kit, according to manufactures protocol with some modifications. The immunoblot in the upper panel in figure 33 (i) was probed for Nrf3 after Nrf2 was pulled down. The same blot was stripped and probed for Nrf2 shown in the lower panel of figure 33 (ii). The immunoblot in the upper panel in figure 33 (iii) was probed for Nrf2 after Nrf3 was pulled down. The same blot was stripped and probed for Nrf3 shown in the lower panel of figure 33 (iv).

4.2.5. Res-analogs significantly induce Nrf2 total mRNA and protein.

The effects of TIMBD and HPIMBD on the regulation of Nrf2 mRNA and protein expression levels in MCF-10A cells were examined. 50 μM concentrations of TIMBD significantly increased Nrf2 mRNA (Figure 34 A) expression levels at 24 hour; and Nrf2 protein (Figure 34 B) expression levels at 48 hour in MCF-10A cells. 50 μM concentrations of HPIMBD also significantly increased Nrf2 mRNA (Figure 34 D) expression levels at 24 hour; and Nrf2 protein (Figure 34 E) expression levels at 48 hour in MCF-10A cells. The fold changes in Nrf2 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res and 50 μM TIMBD for 48 hour were 0.52, 2.33, and 3.97, respectively, compared to vehicle-treated control cells (Figure 34 C). The fold changes in Nrf2 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res and 50 μM HIMBD for 48 hour were 0.52, 2.33, and 3.24, respectively, compared to vehicle-treated control cells (Figure 34 F).

(A)

(B)

(C)

(D)

(E)

(F)
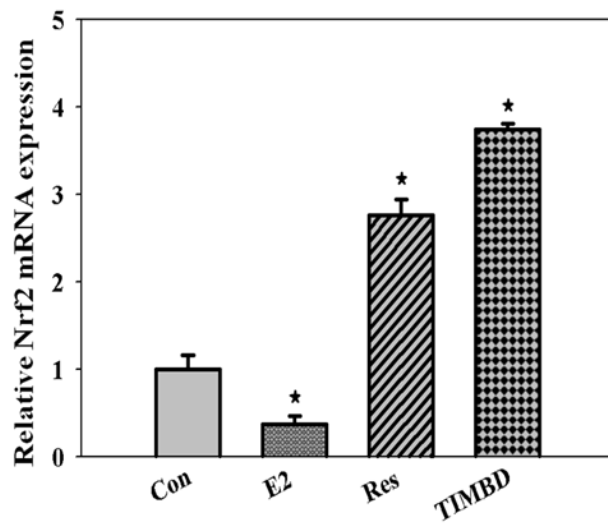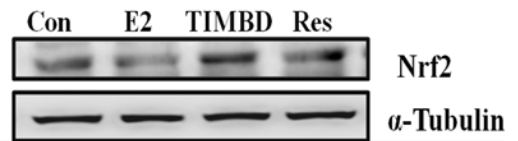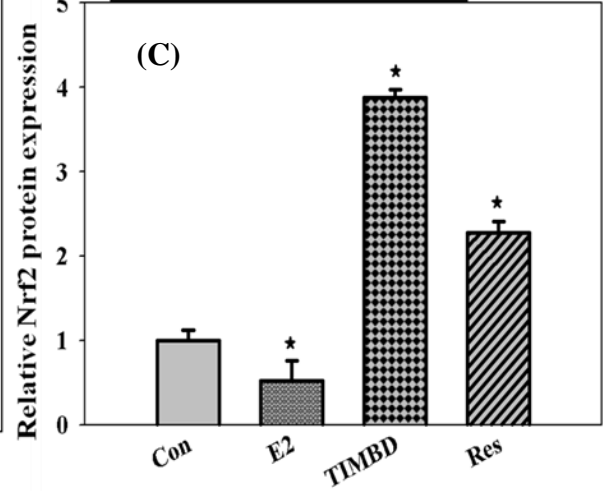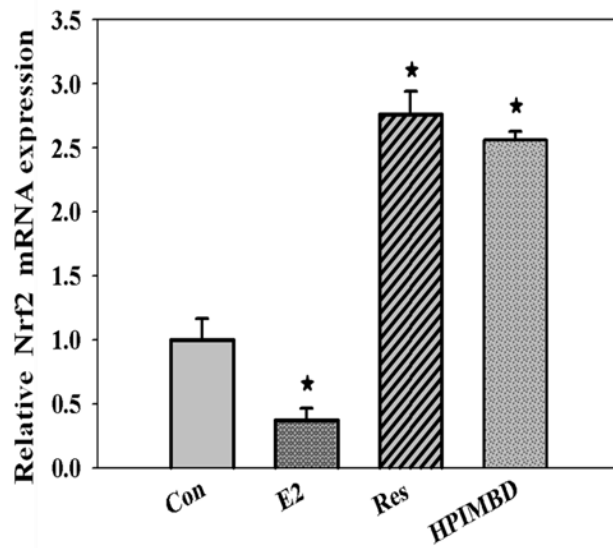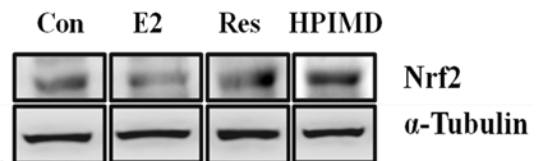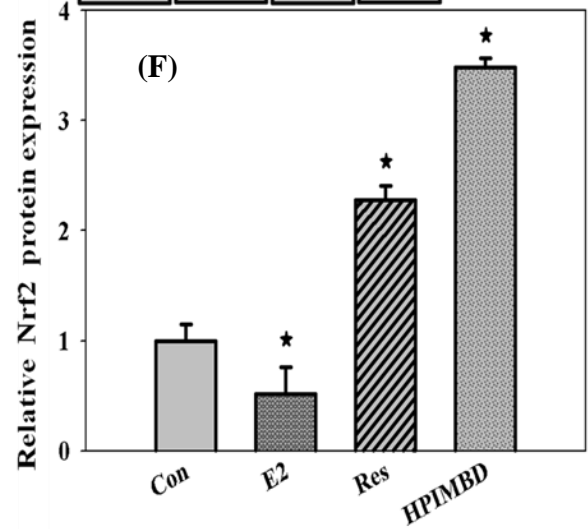
Figure 34: 17 β-Estradiol (E2) down-regulates, whereas Res-analogs reverse E2-mediated decrease in Nrf2 mRNA and protein expression levels, and further increase its mRNA and protein expression levels in MCF-10A cells. (A) Nrf2 mRNA expression levels in MCF-10A cell line treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for 24 hour; (B) Representative Western blot showing up-regulation of Nrf2 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for upto 48 hour; (C) Relative Nrf2 protein expression levels in MCF-10A cell line treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for upto 48 hour; (D) Nrf2 mRNA expression levels in MCF-10A cell line treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for 24 hour; (E) Representative Western blot showing up-regulation of Nrf2 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for upto 48 hour; and (F) Relative Nrf2 protein expression levels in MCF-10A cell line treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for upto 48 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.

4.2.6. Res-analogs reverse E2-mediated up-regulation of Nrf3 mRNA and protein.

The effects of TIMBD and HPIMBD on the regulation of Nrf3 mRNA and protein expression levels in MCF-10A cells were examined. MCF-10A cells were treated with E2 (50 nM), 50 µM of Res, TIMBD or HPIMBD for upto 72 hour. Treatment with E2 significantly increased Nrf3 mRNA expression levels at 48 hour and protein expression levels at 72 hour, compared to control (Figure 35 A & 35 B). Treatment with TIMBD reversed E2-mediated increase in Nrf3 mRNA (Figure 35 A) as well as protein expression levels (Figure 35 B), and significantly decreased Nrf3 mRNA and protein expression levels in MCF-10A cells compared to controls (Figure 35 A & 35 B). Treatment with HPIMBD also reversed E2-mediated increase in Nrf3 mRNA (Figure 35 D) as well as protein expression levels (Figure 35 E), and significantly decreased Nrf3 mRNA and protein expression levels in MCF-10A cells compared to controls (Figure 35 D & 35 E). Resveratrol-mediated decrease in Nrf3 mRNA (Figure 35 A & 35 D) and protein (Figure 35 C & 35 F) expression levels at 24 hour time point was not noticed at 48 hour time point, where as TIMBD and HPIMBD-mediated decrease in Nrf3 protein expression was significant at 48 hour as compared to control. The fold changes in Nrf3 protein expression levels in MCF-10A cells treated with E2, Res or TIMBD for 48 hour were 2.93, 0.89 and 0.33 respectively, compared to vehicle-treated control cells (Figure 35 C). The fold changes in Nrf3 protein expression levels in MCF-10A cells treated with E2, Res or HPIMBD for 48 hour were 2.93, 0.89 and 0.41 respectively, compared to vehicle-treated control cells (Figure 35 F).
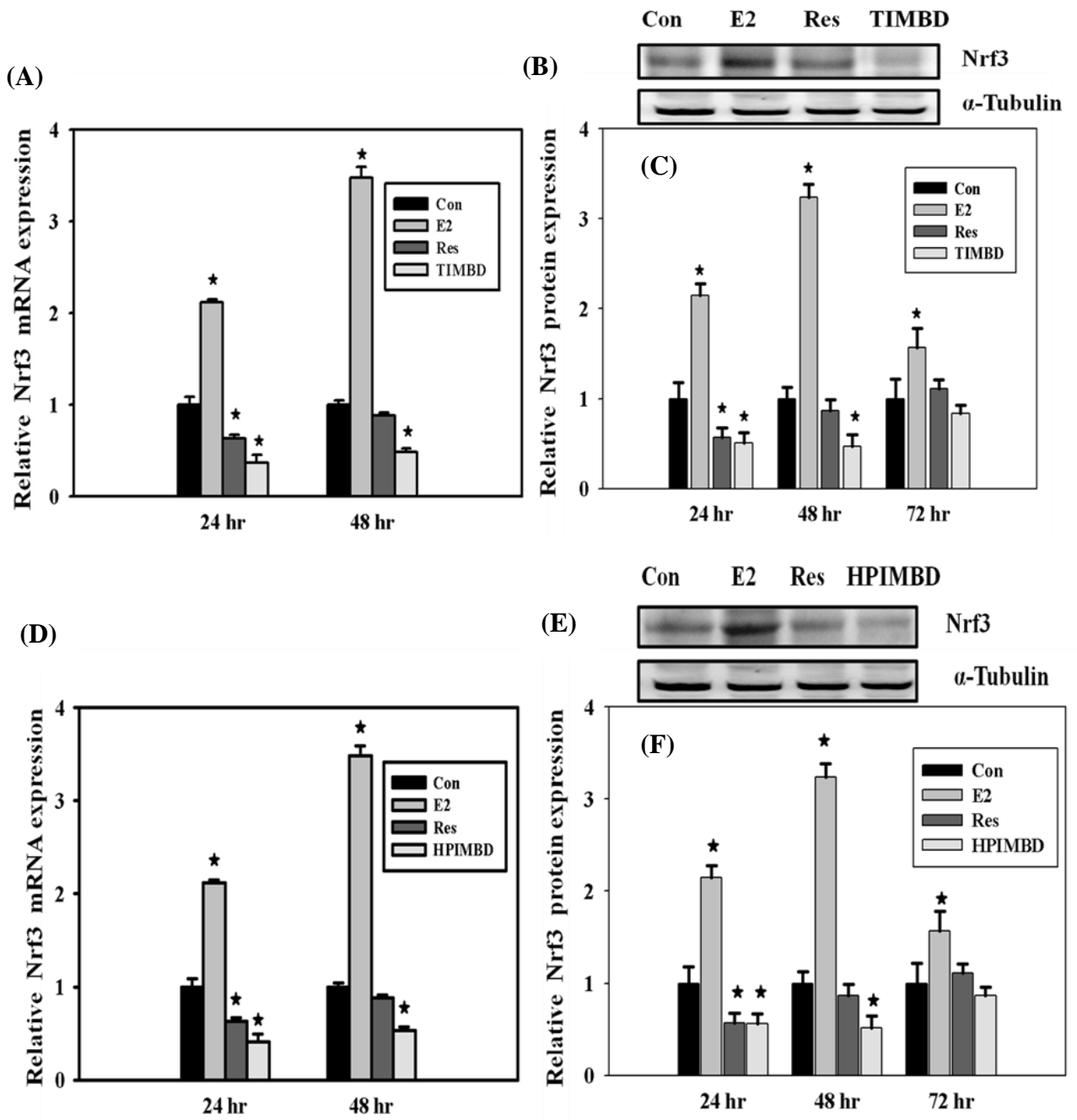
Figure 35: 17 β-Estradiol induces Nrf3 mRNA and protein expression levels in MCF-10A cells. Res-analogs, TIMBD or HPIMBD reverse E2-mediated increase in Nrf3 mRNA and protein expression levels in MCF-10A cells. (A) Nrf3 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for upto 48 hour; (B) Representative Western blot showing reversal of E2-mediated up-regulation of Nrf3 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for upto 48 hour; (C) Relative Nrf3 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for upto 72 hour; (D) Nrf3 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for upto 48 hour; (E) Representative western blot showing reversal of E2-mediated up-regulation of Nrf3 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for upto 48 hour; and (F) Relative Nrf3 protein expression levels in MCF-10A cell line treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for upto 72 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.

4.2.7. Res-analogs up-regulate Nrf1 mRNA and protein in MCF-10A cells.

Nrf1 mRNA and protein expression levels were quantified in MCF-10A cells treated with E2 (50 nM) for upto 72 hour. A significant increase in Nrf1 mRNA as well as protein expression levels compared to control was detected (Figure 36 A & 36 B). Resveratrol-analog HPIMBD by itself significantly increased Nrf1 mRNA and protein expression levels compared to vehicle-treated control cells (Figure 36 A & 36 B). Resveratrol-analog TIMBD also by itself significantly increased Nrf1 mRNA and protein expression levels compared to vehicle-treated control cells (Figure 36 D & 36 E). This increase of Nrf1 was independent of E2 treatment. The fold changes in Nrf1 protein expression levels in MCF-10A cells treated with E2, Res and HPIMBD for 24 hour were 3.13, 2.73, and 2.88, respectively, compared to vehicle-treated control cells (Figure 36 C). The fold changes in Nrf1 protein expression levels in MCF-10A cells treated with E2, Res and TIMBD for 24 hour were 3.10, 2.62, and 2.95, respectively, compared to vehicle-treated control cells (Figure 36 F).

(A)

**(B)** Con E2 Res HPIMBD — Nrf1 / α-Tubulin

(C)

(D)

(E) Con E2 Res TIMBD — Nrf1 / α-Tubulin

(F)
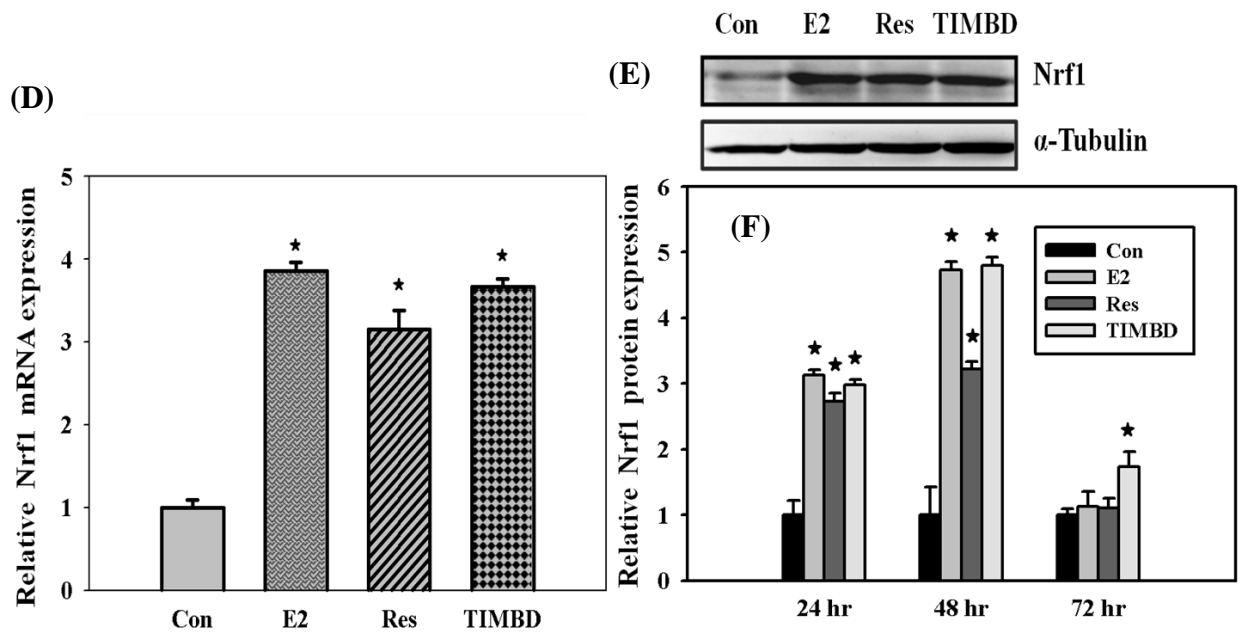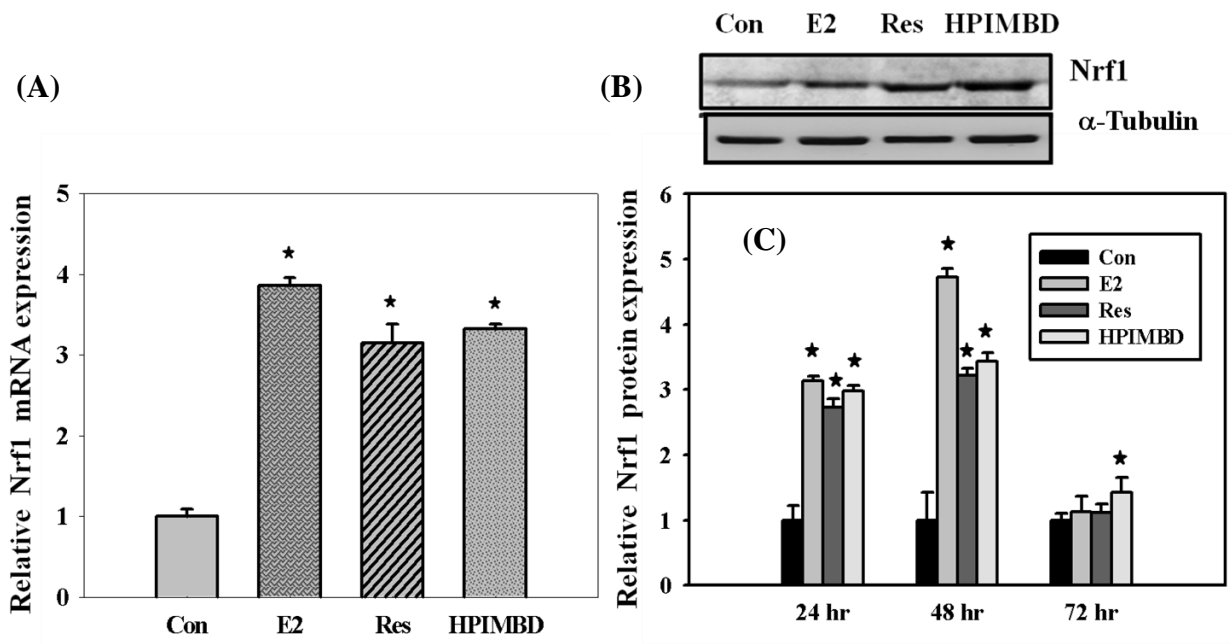
Figure 36: 17 β-Estradiol induces Nrf1 mRNA and protein expression levels in MCF-10A cells. Res-analogs, TIMBD or HPIMBD induce Nrf1 mRNA and protein expression levels in MCF-10A cells. (A) Nrf1 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for 24 hour; (B) Representative western blot showing up-regulation of Nrf1 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for 24 hour; (C) Relative Nrf1 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for upto 72 hour; (D) Nrf1 mRNA expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for 24 hour; (E) Representative western blot showing up-regulation of Nrf1 protein expression levels in MCF-10A cells treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for 24 hour; and (F) Relative Nrf1 protein expression levels in MCF-10A cell line treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for upto 72 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.

4.3 Discussion:

Cap'n'collar (CNC) basic leucine zipper transcription factors are well known for their crucial roles in regulation of mammalian genes involved in developmental and homeostatic processes (150-153). Among the vertebrate CNC family of proteins, the most studied transcription factors are Nuclear factor erythroid 2-related factor 1 (Nrf1, also called NFE2L1), nuclear factor erythroid 2-related factor 2 (Nrf2), and nuclear factor erythroid 2-related factor 3 (Nrf3), known for their crucial roles in providing adaptive responses to cellular stresses (150-153). The most extensively explored CNC family of transcription factor is Nrf2 which regulates expressions of genes in response to elevated cellular oxidative stress and keeps the mediators of oxidative stress in control (154). In presence of oxidative stress mediators when reactive oxygen species (ROS) are produced in the cells, as a defense response to prevent excessive ROS mediated damages and keep the oxidative stress in check, Nrf2 levels are elevated. The nuclear factor erythroid 2-related factor (Nrf2) is a master regulator which is responsible for transcriptional activation of a variety of antioxidant genes and phase-II detoxifying enzymes. These antioxidant genes in turn bring back cellular redox homeostasis. Under normal cellular conditions when no oxidative stress is encounterd, Nrf2 remains sequestered in the cytosol by an adaptor protein named Kelch-like ECH-associated protein 1 (Keap1). Keap 1 is cysteine-rich protein that acts as a negative regulator of Nrf2 by interacting to it and keeping it sequestered in the cytosol. Under normal conditions Keap1 mediates rapid proteasomal-degradation of Nrf2 as a result of its ubiquitination and maintains the cellular base level of Nrf2 (158). When oxidative stress is encountered, cysteine residues of Keap1 act as sensor and detect changes in cellular redox state. Under such

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circumstances Keap1 fails to bind to Nrf2 and promote its proteasomal-degradation. As a result, unbound and free Nrf2 gets dissociated from its negative counterpart Keap 1 and translocates to the nucleus to turn on antioxidant gene expressions (155). This interaction between Keap1 and Nrf2 is not steady, it takes place only when there is an elevated oxidative stress encountered in the cell and this interaction takes place inside the nucleus after Keap1 is shuttled in (155). Inside the nucleus Nrf2 forms heterodimer by binding to small Maf proteins and this heterodymeric form binds to the antioxidant response elements (ARE) of the DNA of antioxidant and Phase-II detoxifying genes resulting in their transcriptional activation.

The Nrf2 signaling pathway is responsible for mediating cellular defense against oxidative burden and a variety of detoxifying and cytoprotective genes are regulated by this master transcription factor (158). Different antioxidant proteins and Phase-II detoxifying enzymes that play key roles in scavenging excessive ROS production and provide cellular defense against oxidative damage to the cell includes superoxide dismutases, glutathione peroxidase, heme oxygenase, flavin containing monooxygenase 1, NQO1 etc (36, 49, 165-167). Recent studies from our laboratory and that of others have shown critical role of Nrf2 in E2-induced breast carcinogenesis (167-170). Since Nrf2 regulates several antioxidant enzymes and our Res-analogs TIMBD and HPIMBD have shown to significantly up-regulate Phase-II detoxifying enzymes SOD3 and NQO1, we wanted to examine if our novel Res-analogs also regulate Nrf2 expression in breast epithelial cells. In this study we found significant suppression of Nrf2 mRNA and protein expression levels in E2-treated MCF-10A cells (Figure 30 A-D). This suppression was reversed and further increased upon co-treatment with prototypic antioxidants Res or VC

(Figure 30E & 30 F), or Res-analogs (Figure 34). Treatment with TIMBD significantly increased the mRNA and protein expression levels of Nrf2 better than Res (Figure 34 A & 34 B). Treatment with HPIMBD also significantly increased the mRNA and protein expression levels of Nrf2 (Figure 34 D & 34 E). These findings suggested that Res, TIMBD and HPIMBD regulate E2-induced breast cancer via Nrf2-dependant signaling pathway.

Nuclear factor erythroid 2-related factor 1 (Nrf1, also called NFE2L1) is another member of CNC b-zip protein family. Nrf1 is well known to regulate expression of cytoprotective genes through binding to the antioxidant-response elements in the promoter regions of these genes (159, 160). Examples of cytoprotective genes those are transcribed by Nrf1 include NAD(P)H:quinone oxidoreductase-1 (NQO1), Heme Oxygenase 1 (HMOX-1), Metallothionein, Ferritin etc (152, 153). The well studied functions of Nrf1 include prevention of oxidative stress, prevention of metal-induced toxicity and induction of anti-apoptotic genes from bcl2 family (159, 162). Nrf1 is suggested to play critical role in embryonic development; it has been shown that *Nrf1-/-* mice die prematurely due to anemia (153). But unlike Nrf2, Nrf1 is not that well studied for its role in prevention of oxidative stress and estrogen-induced breast cancer. Thus in this study we wanted to examine the role of Nrf1 in prevention of E2-induced breast cancer and the effects of Res, and Res-analogs in its regulation. We found significant increase in Nrf1 mRNA and protein expression following E2 treatment in MCF-10A cells (Figure 31 C & 31 D). Treatment with Res or VC (Figure 31 C & 31 D), or Res-analogs (Figure 36) also increased Nrf1 mRNA and protein expression levels. From these results it could be suggested that Nrf1 has compensatory role in E2-induced stress and Res;

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TIMBD and HPIMBD play chemopreventive role in E2-induced breast cancer by up-regulation of cytoprotective gene Nrf1. Nevertheless, more studies are needed to be done to show specific role of Nrf1 in E2-induced breast cancer.

Nuclear factor erythroid-derived 2-like 3 (Nrf3) is also a CNC-bZIP transcription factor (150, 152). It is suggested that Nrf3 plays important role in a variety of cellular processes including. inflammation, differentiation and carcinogenesis. Till date there is no study suggesting important role of Nrf3 in E2-induced breast cancer. In this study we examined the role of Nrf3 in E2-induced breast cancer and the effect of prototypic antioxidants Res, and Res-analogs TIMBD and HPIMBD in regulation of Nrf3. We found that E2 treatment increased Nrf3 mRNA and protein expression levels in a time dependant manner in MCF-10A cells (Figure 31 A & 31 B). Estrogen-mediated increase in the expression of Nrf3 mRNA and protein was reversed by antioxidants VC or Res (Figure 31 A & 31 B). This Estrogen-mediated increase in Nrf3 mRNA and protein levels was also reversed by our novel Res-analogs (Figure 35). These results further support the importance of Nrf3 in E2-induced breast cancer. These results suggest that since Nrf3 is a negative regulator of antioxidant response, VC-, Res- or Res-analogs-mediated suppression of Nrf3 may protect the cellular phase-II detoxifying enzymes and mediate protection to the normal breast tissue from carcinogenic insult by E2.

The three Nrfs have broad and partly overlapping expression patterns and functions as stress-activated transcription factors. Nrf3 is the third member of the Nrf gene family (152). Nrf3 is known to repress NQO1 gene expression and to negatively regulate antioxidant response (164). In order to better understand the mechanisms by which Nrf3 regulated in E2-induced breast cancer we examined the possible relationship

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between Nrf2 and Nrf3. The co-existence of Nrf3 with Nrf2 shown by Co-Ip assay and the increased expression of Nrf2 and NQO1 following silencing of Nrf3 suggests that probably Nrf2 is regulated by Nrf3 (171). Further studies are needed to be done to establish this relationship between Nrf2 and Nrf3.

Since Nrf1, Nrf2 and Nrf3 control phase II detoxification enzymes that help in detoxification of potential carcinogens, understanding the mechanisms of regulation of these three "Nrfs" may help in the development of cancer therapeutics. We report in this study that antioxidants mediate their protective role in E2-induced breast cancer through a complex interplay of CNC b-zip family of proteins.

CHAPTER 5

DETERMINATION OF THE ROLE OF RES-ANALOGS IN EPITHELIAL-

MESENCHYMAL TRANSITION (EMT) IN BREAST CANCER

5.1. Introduction

Epithelial–mesenchymal transition (EMT) is a biological process which allows epithelial cells to undergo various molecular changes and to assume mesenchymal-like phenotype (172). This process results in changes in functional characteristics of the cells which become more migratory type (mesenchymal) than that of polarized type epithelial cells likely to interact with the basement membrane (172). Epithelial–mesenchymal transition is a key step during embryogenesis and also implicated in tissue regeneration (173). But an excess of epithelial cell proliferation and migration has fatal consequences of cancer metastasis (173). Following transformation to mesenchymal phenotype, the epithelial cancer cells acquire migratory and invasive properties, invade through the basement membrane, and eventually metastasize with life-threatening consequences. Determination of molecular, biochemical, genetic and epigenetic basis of EMT and cancer metastasis has been very active field of research (174). A number of these studies suggest that activation of an EMT program is a critical mechanism for the transformation of epithelial cancers to malignant and metastatic types (175). One of the most common epithelial cancers are breast cancers, breast cancer metastasis is also largely known to be consequences of the EMT program (176). The molecular mechanism of EMT has been well characterized (177-179). One most important characteristic molecular feature of

EMT is decrease in the expression of epithelial cell marker, E-cadherin. E-cadherin is a transmembrane protein that plays very important role in cell adhesion, cell-cell and cell-matrix interactions (180). Decreased E-cadherin expression in epithelial tumor cells implies onset of metastasis (181). Without E-cadherin the cells lose their epithelial characteristics. Like other cancers, breast carcinogenesis is also associated with the decreased function and expression levels of E-cadherin (182). At the molecular level, E-cadherin expression is controlled by quite a few transcription factors related to the process of EMT, like, snail, slug, zeb 1, and zeb 2 (183, 184). These transcription repressors of E-cadherin have traditionally been implicated in promoting EMT.

Different studies are suggestive of the fact that E-cadherin is an invasive suppressor and its loss of expression is associated with poor prognosis in breast cancer. Thus, targeting inhibition of the process of EMT program may help in developing strategy for the prevention of breast cancer cell metastasis. Resveratrol has been well recognized as a potential cancer chemopreventive agent. The potential role of Res in inhibition of EMT in breast cancer has been well established (185). Resveratrol suppresses EMT by modulating different signaling pathways (185, 186). As discussed before in chapter-3, in spite of being potent anti-tumorigenic agent *in vitro,* Res has its own limitations of poor efficacy in human body that stops it to be used as a preferable drug of cancer chemotherapy. Thus, to improve the anticancer efficacy of Res, we have synthesized aza-resveratrol analogs: TIMBD and HPIMBD. Our previous studies as discussed in the previous chapters, demonstrate that the novel Res-analogs inhibit the growth of different human cancer cells and have no toxicity towards non-tumorigenic human breast epithelial cells. However, the effects of TIMBD and HPIMBD on the

metastasis of breast cancer cells have not been elucidated. To address this issue, we initially assessed the expression profiles of EMT-related markers in very aggressive metastatic breast cancer cells, MDA-MB-231 after treatment with Res, TIMBD or HPIMBD.

Matrix metalloproteinases (MMPs) are enzymes that break down and modify the extracellular matrix resulting in decreased cell-cell and cell-matrix contacts (187). As a consequence, epithelial cells get detached from the surrounding tissue and metastasize (187). MMPs are well known to play key role in embryonic development and organ morphogenesis (188, 189). Recent studies suggest that MMPs also play important role in cancer metastasis (190). MMPs are frequently found to be up regulated in breast cancer, facilitating tumorigenesis, cancer cell invasion and migration (191). In this current study, we have examined the regulation of MMPs by Res-analogs in metastatic breast cancer cells.

Our study demonstrated that Res-analogs TIMBD and HPIMBD are more effective than the parent compound Res in inducing E-cadherin and down-regulation of EMT-inducers: MMPs, Snail, Slug and Zeb1. All these changes in molecular markers of EMT imply inhibition of EMT. Furthermore, we have demonstrated that our Res-analogs are very potent to inhibit the migratory properties of MDA-MB-231 cells by performing wound healing assay. To further delineate the mechanism of action of Res-analogs in inhibition of the process of EMT, we found involvement of wnt/β-catenin signaling pathway which is implicated to drive the process of EMT in carcinogenesis. Our results demonstrate the potential of our novel Res-analogs in preventing the EMT-induced

malignant transformation of breast carcinoma through modulation of wnt/β-catenin pathway.

5.2. Results

5.2.1. Res-analogs down regulate Matrix metallo proteinases (MMPs) in metastatic breast cancer cells.

Matrix metallo proteinases (MMPs) are very important enzymes responsible for extracellular matrix (ECM) breakdown and remodeling of epithelial mesenchyme. ECM breakdown is the ultimate step leading towards invasion, migration and metastasis of cancer cells. In this study, we have examined the protecting effects of Res, TIMBD and HPIMBD against breast cancer metastasis. We have tested the effects of our Res-analogs in the protein expression levels of MMP2, MMP3 and MMP9 in metastatic breast cancer cells, MDA-MB-231, treated for upto 48 hour. TIMBD inhibited MMP 2, -3, and -9 significantly (Figure 37 A). TIMBD, but not Res, inhibited the protein expression levels of MMP 2 and -9 in MDA-MB-231 cells at its IC_{50} dose (21μM) (Figure 37 A). HPIMBD, on the other hand, inhibited the protein expression levels of MMP 3 and -9; but not MMP 2 in MDA-MB-231 cells at its IC_{50} dose (29 μM) (Figure 37 B). Resveratrol was only able to down regulate MMP 3 at its IC_{50} dose (66 μM) (Figure 37 A & 37 B).

(A)   **(B)**
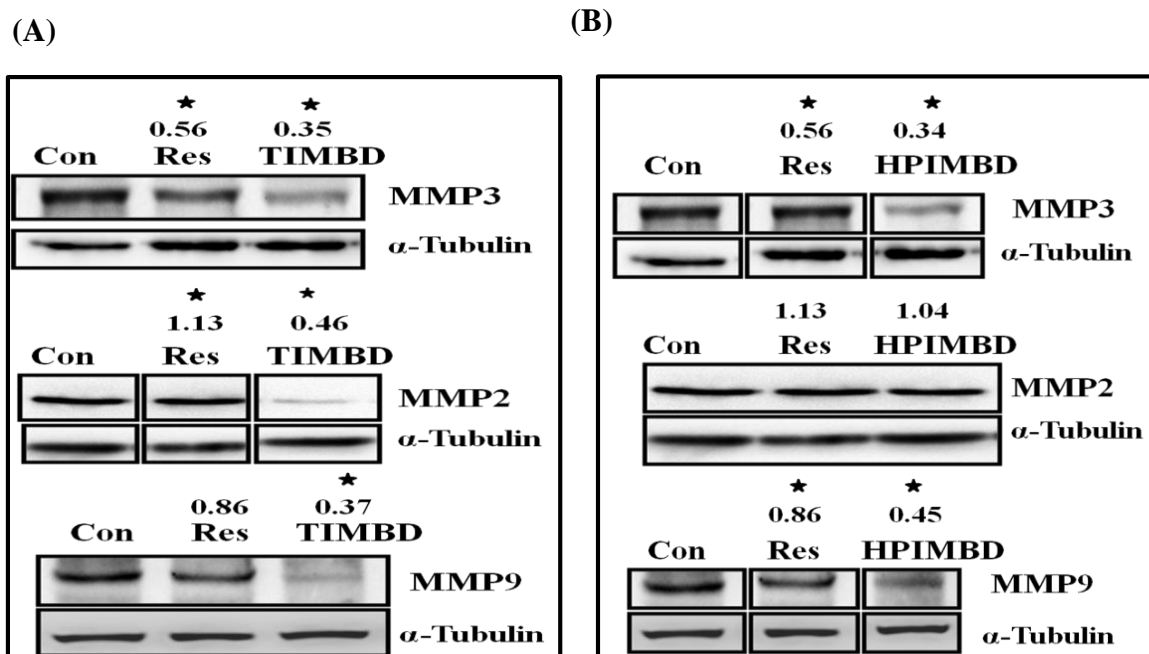
Figure 37: Res-analogs, TIMBD and HPIMBD down-regulate matrix metallo proteinases (MMPs) in metastatic breast cancer cells, MDA-MB-231. (A) Representative western blot showing protein expression levels of MMP 3, MMP 2 and MMP 9 in MDA-MB-231 cells treated with IC_{50} doses of Res (66 µM) or TIMBD (21µM) for upto 48 hour. (B) Representative Western blot showing protein expression levels of MMP 3, MMP 2 and MMP 9 in MDA-MB-231 cells treated with IC_{50} doses of Res (66 µM) or HPIMBD (29 µM) for upto 48 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.

5.2.2. Mechanism of inhibition of EMT by Res-analogs in metastatic breast cancer cells.

To elucidate the role of Res-analogs in the EMT program of breast carcinogenesis, we examined the effect of the Res-analogs TIMBD and HPIMBD on the expression of EMT-representative markers in MDA-MB-231 metastatic breast cancer cells by Western blot analysis and compared these effects with the parent compound Res. Our results demonstrate that at their IC_{50} concentrations of 66 μM, 29 μM and 21 μM, respectively for Res, HPIMBD and TIMBD, resulted in an elevated expression of epithelial marker E-cadherin, and consequently repressing the expression of mesenchymal-related markers snail, slug and zeb1 (Figure 38 A & 38 B). However, Res, TIMBD and HPIMBD had no effects on the expression level of mesenchymal-related marker zeb 2 (Figure 38 A & 38 B). Our results show that TIMBD and HPIMBD were better effective than Res in up-regulation of E-cadherin; the fold changes in E-cadherin protein expression levels in MDA-MB-231 cells treated with Res, TIMBD and HPIMBD for 24 hour were 2.12, 3.11 and 2.55 , respectively, compared to vehicle-treated control cells (Figure 38 A & 38 B). Among the two Res-analogs, TIMBD was more effective in down-regulation of suppressors of E-cadherin; snail and zeb 1. The fold changes in snail protein expression levels in MDA-MB-231 cells treated with Res, TIMBD and HPIMBD for 24 hour were 0.42, 0.41, 0.53. respectively, compared to vehicle-treated control cells (Figure 38 A & 38 B).. The fold changes in zeb 1 protein expression levels in MDA-MB-231 cells treated with Res, TIMBD and HPIMBD for 24 hour were 0.46, 0.41, 0.45 respectively, compared to vehicle-treated control cells (Figure 38 A & 38 B). Although TIMBD and HPIMBD significantly and effectively down-regulated slug protein

expression levels, however, Res-mediated down regulation of slug protein expression was better than Res-analogs TIMBD and HPIMBD. The fold changes in slug protein expression levels in MDA-MB-231 cells treated with Res, TIMBD and HPIMBD for 24 hour were 0.27, 0.31 and 0.56, respectively, compared to vehicle-treated control cells (Figure 38 A & 38 B).
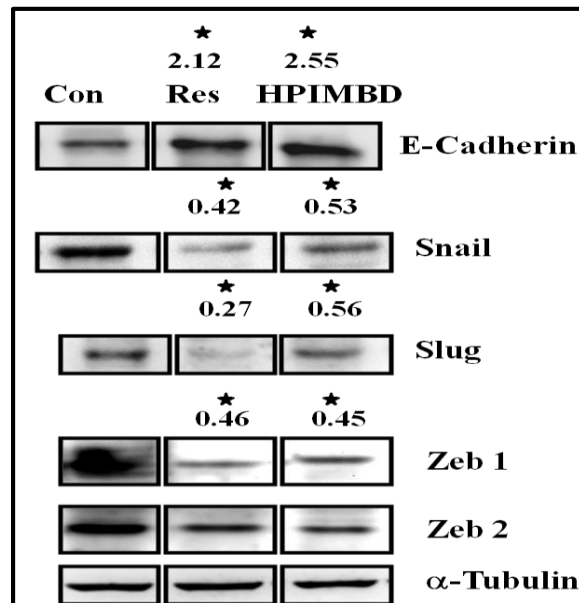
Figure 38: Mechanism of regulation of E-Cadherin by Res-analogs, TIMBD and HPIMBD in metastatic breast cancer cells, MDA-MB-231. (A) Representative western blot showing significant up-regulation of E-cadherin protein; and down-regulation of repressors of E-cadherin: Snail, Slug and Zeb 1 protein expression levels in MDA-MB-231 cells treated with IC_{50} doses of Res (66 μM) or TIMBD (21μM) for upto 48 hour. (B) Representative western blot showing significant up-regulation of E-cadherin protein; and down-regulation of repressors of E-cadherin: Snail, Slug and Zeb 1 protein expression levels in MDA-MB-231 cells treated with IC_{50} doses of Res (66 μM) or HPIMBD (29μM) for upto 48 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.

5.2.3. Res-analogs inhibit EMT through regulation of Wnt/β-catenin pathway in breast cancer.

β-catenin functions as a transcriptional coactivator of the canonical Wnt signaling pathway, which contributes to EMT during tumorigenesis (192, 193). The dissociation of β-catenin from the cytoplasmic tail of E-cadherin may increase the nonjunctional pool of β-catenin in cytosol, thereby leading to epithelial cell migration (194). Therefore, to elucidate whether the increase in E-cadherin in response to our novel Res-analogs TIMBD and HPIMBD in MDA-MB-231 cells is accompanied by the reconstitution of the E-cadherin–β-catenin complex and the suppression of Wnt/β-catenin signaling, the expression, cellular distribution, and function of β-cate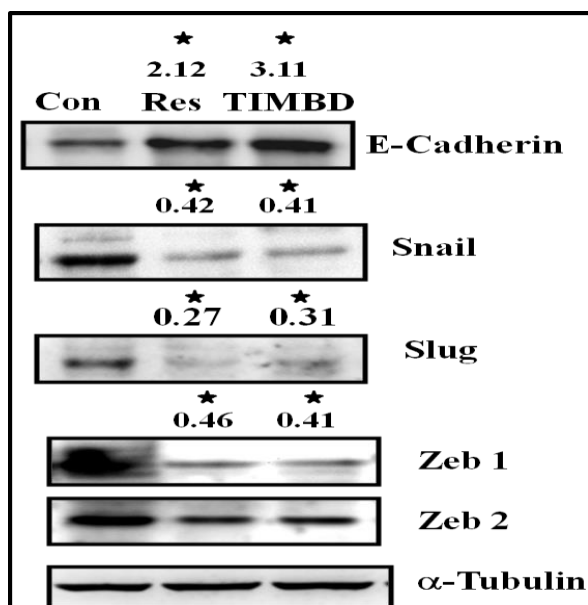nin were studied. An inhibition of β-catenin mRNA levels was observed in the Res-analogs treated MDA-MB-231 cells at a dosage of 50 µM (Figure 39 B & 39 C). Consistently, TIMBD and HPIMBD reduced the nuclear translocation of β-catenin protein (Figure 41 A & 41 B). In particular, the knockdown of β-catenin by specific siRNA converted the expression status of the EMT marker E-cadherin in MDA-MB-231 cells compared with that of the scramble siRNA control (Figure 42). Subsequently, colony formation assay was performed in MDA-MB-231 cells transfected with si- β-catenin to determine growth promoting effect of β-catenin. The results show that si-β-catenin treatment decreased the cancer cell colonies suggesting tumorigenic role of β-catenin in metastatic breast cancer cell MDA-MB-231 (Figure 39 A). Down-regulation of Frizzled-4 mRNA (Figure 39 D & 39 E, and Wnt 7b mRNA (Figure 39 F & 39 G) was also observed in MDA-MB-231 cells treated with 50 µM of Res, HPIMBD or TIMBD for 24 hour. Resveratrol and its analogs significantly

up-regulated epithelial tumor suppressor gene Wnt 5a in human breast epithelial cells, MCF-10A (Figure 40 A & 40 B). Overall, these results indicate that TIMBD and HPIMBD down-regulates the free pool of nuclear β-catenin, suggesting that the modulation of Wnt/β-catenin signaling may be a possible mechanism of action through which TIMBD and HPIMBD may elicit the EMT reversal of MDA-MB-231 cells.

(A)

Con   E2 50nM   Si-β catenin   Si-β catenin +E2

(B)

(C)

(D)

(E)

(F)

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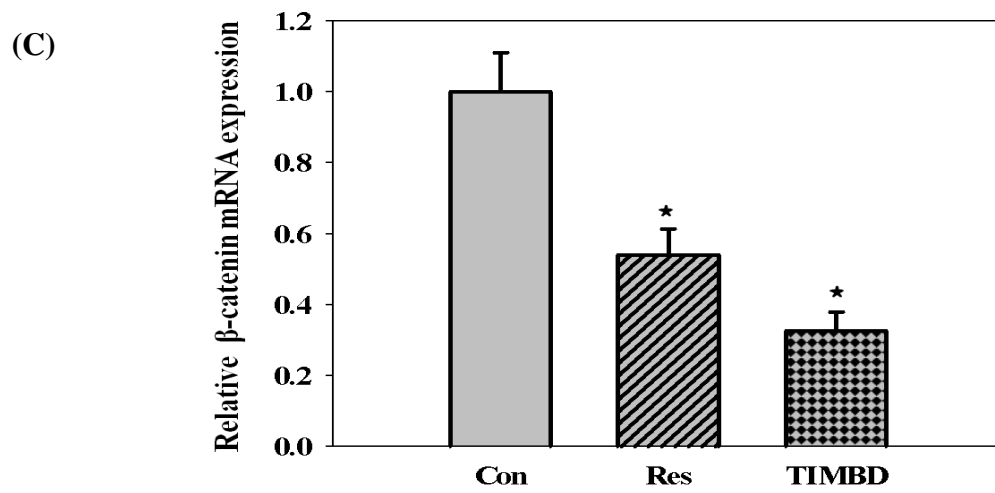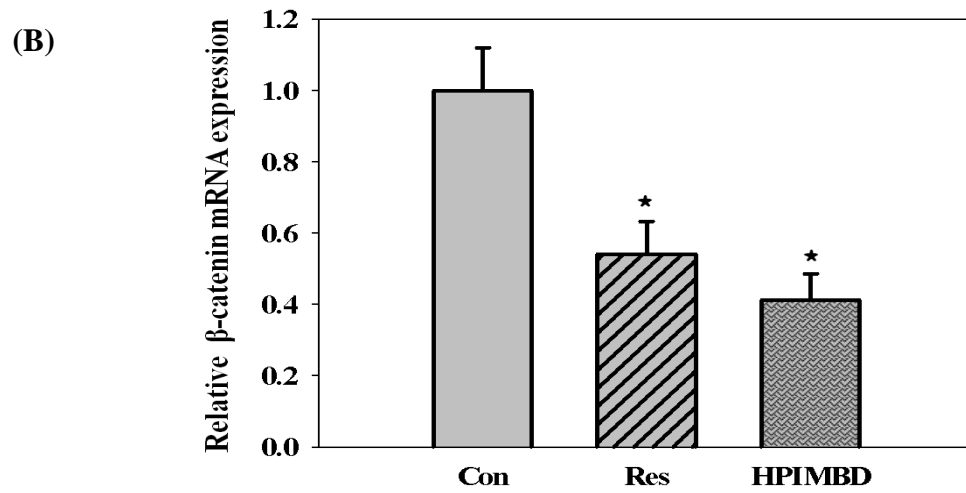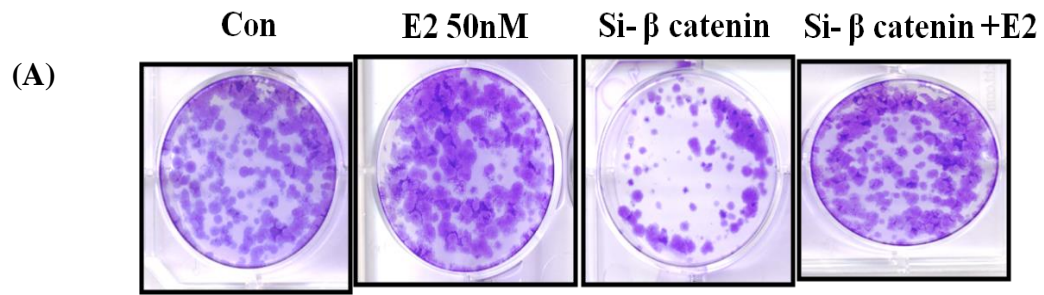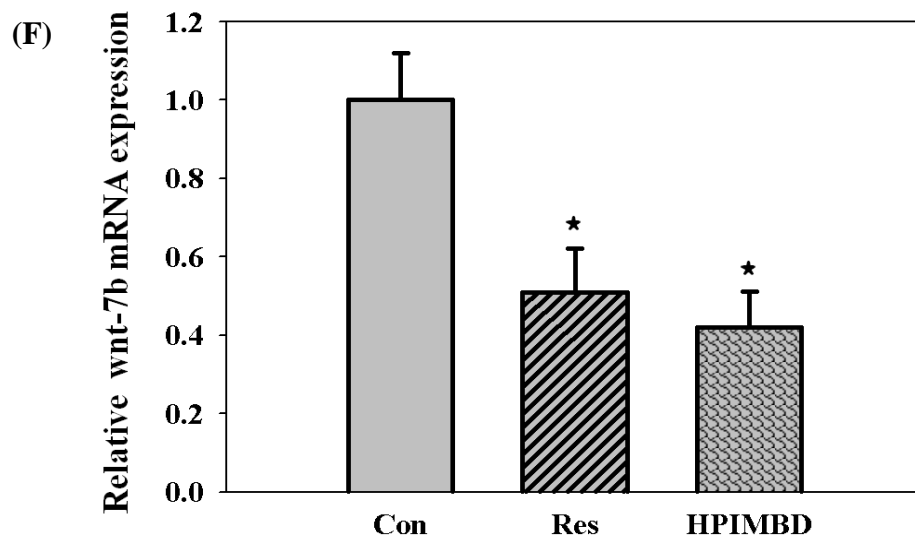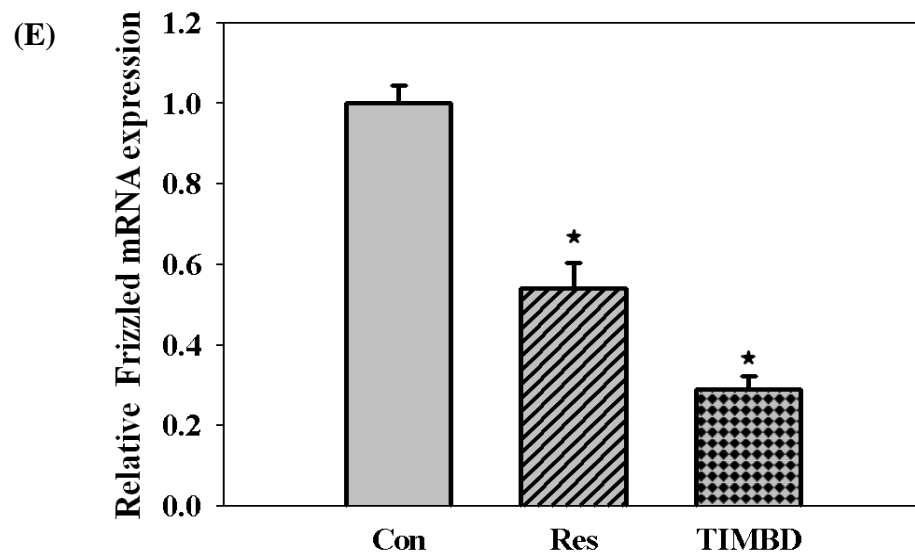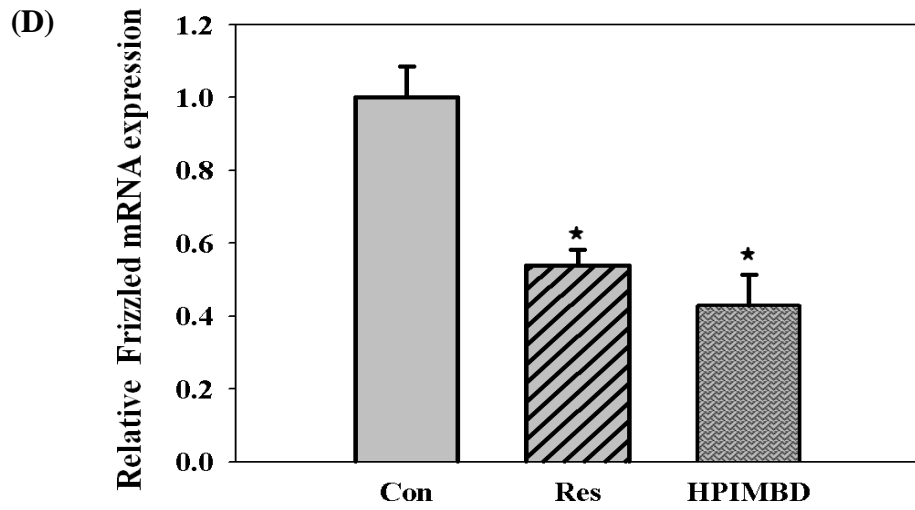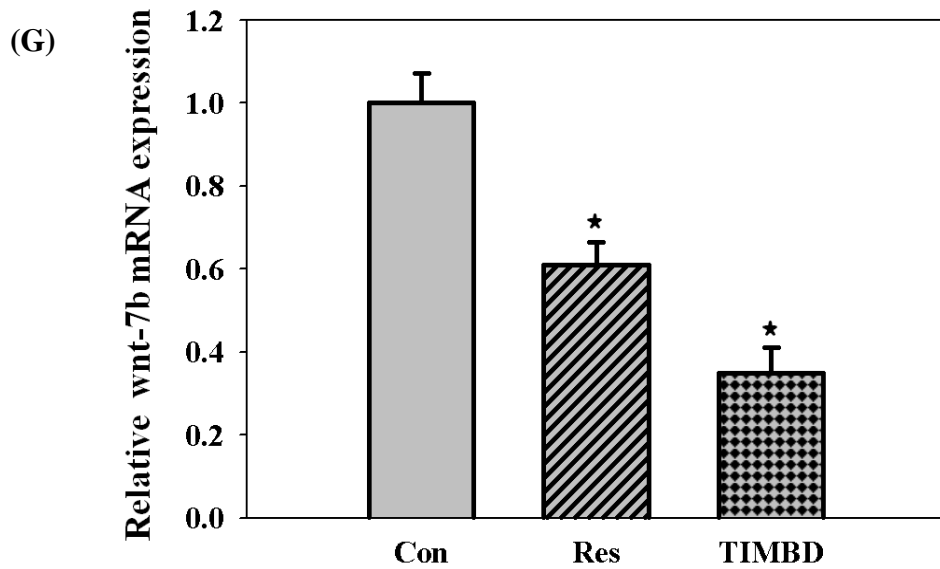
Figure 39: Res-analogs, HPIMBD and TIMBD modulate Wnt/β-catenin signaling in metastatic breast cancer cells, MDA-MB-231. (A) Colony formation assay showing growth promoting effect of β-catenin in MDA-MB-231 cells transfected with si-β-catenin with or without E2; (B) Relative β-catenin mRNA expression in MDA-MB-231 cells treated with 50 μM Res or 50 μM HPIMBD for 24 hour; (C) Relative β-catenin mRNA expression in MDA-MB-231 cells treated with 50 μM Res or 50 μM TIMBD for 24 hour; (D) Relative Frizzled-4 mRNA expression in MDA-MB-231 cells treated with 50 μM Res or 50 μM HPIMBD for 24 hour; (E) Relative Frizzled-4 mRNA expression in MDA-MB-231 cells treated with 50 μM Res or 50 μM TIMBD for 24 hour; (F) Relative wnt-7b mRNA expression in MDA-MB-231 cells treated with 50 μM Res or 50 μM HPIMBD for 24 hour; and (G) Relative wnt-7b mRNA expression in MDA-MB-231 cells treated with 50 μM Res or 50 μM TIMBD for 24 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.

(A)

(B)
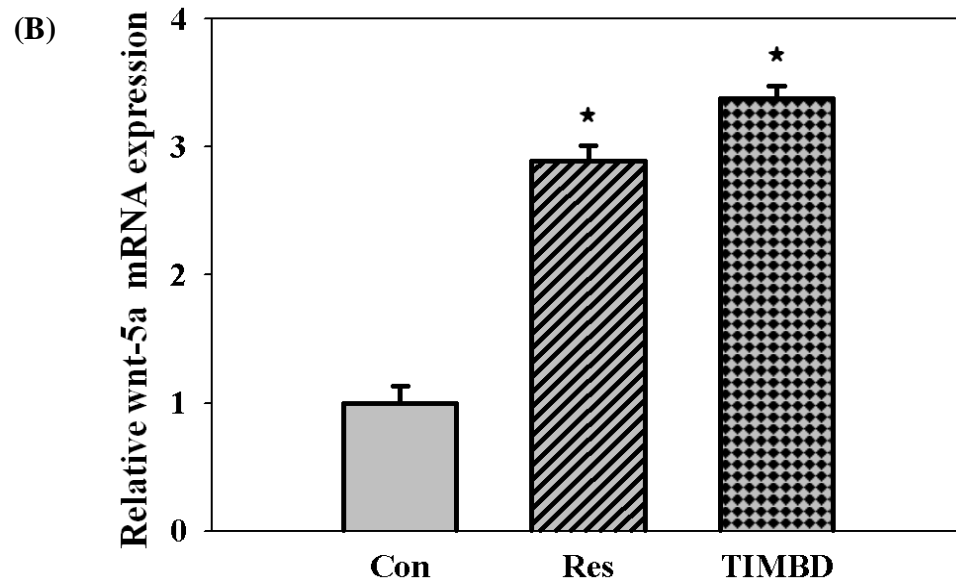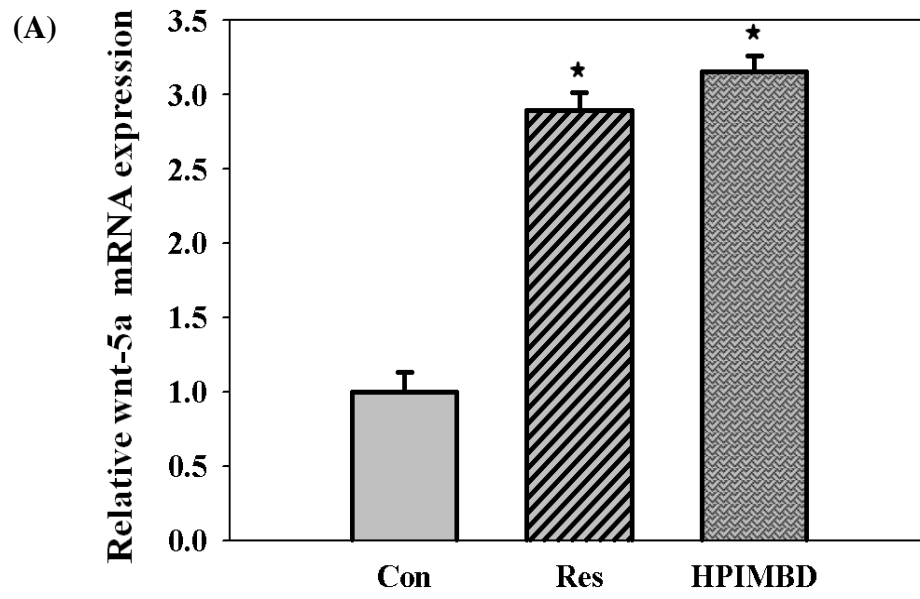
Figure 40: Res-analogs, HPIMBD and TIMBD significantly up-regulate epithelial tumor suppressor gene Wnt-5a in human breast epithelial cells, MCF-10A. (A) Relative Wnt-5a mRNA expression in MCF-10A cells treated with 50 μM Res or 50 μM HPIMBD for 24 hour; and (B) Relative Wnt-5a mRNA expression in MCF-10A cells treated with 50 μM Res or 50 μM TIMBD for 24 hour.

'*' indicates p value ≤ 0.05 compared with respective controls.

5.2.4. Res-analogs inhibit nuclear translocation of β-catenin in breast cancer.

To examine the effects of TIMBD and HPIMBD in distribution of β-catenin protein in the breast cancer cells MDA-MB-231, cytoplasmic and nuclear proteins were extracted from the MDA-MB-231 cells. The levels of β-catenin protein were determined by Western blotting of equal amounts of protein from cytoplasmic and nuclear extracts from MDA-MD-231 cells treated with 66 μM of Res, 29 μM of HPIMBD and 21 μM of TIMBD (IC_{50} values of these compounds for MDA-MB-231 cells) for 6 hour. More β-catenin was found in the cytoplasmic extracts treated with TIMBD or HPIMBD. In contrast, treatment with Res increased nuclear expression of β-catenin (Figure 42) in MDA-MB-231 cells as compared to cytoplasmic extract. These results suggest that our novel Res-analogs TIMBD and HPIMBD are not only capable of down regulation of β-catenin mRNA and protein expression levels, but also they inhibit nuclear translocation of β-catenin protein in breast cancer cells MDA-MB-231.
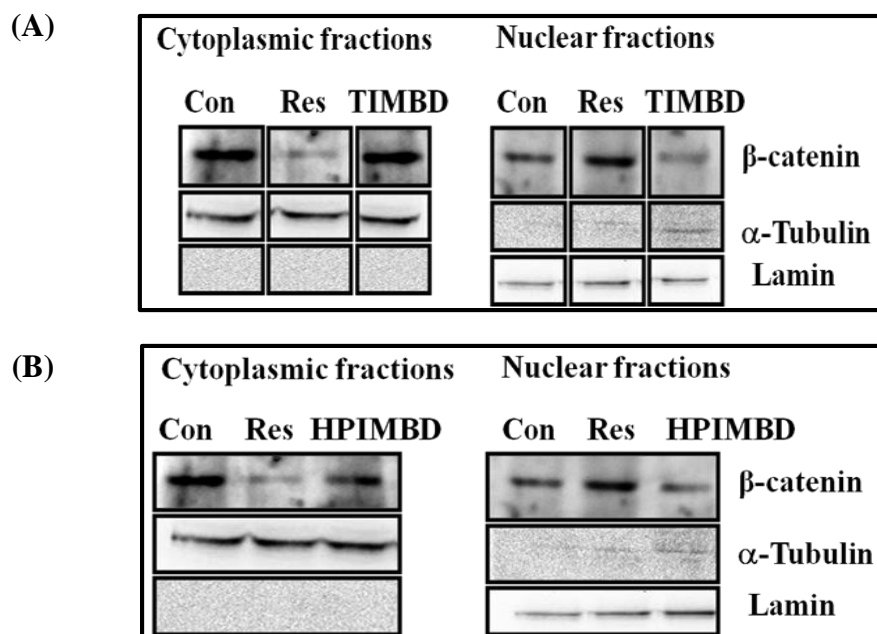
Figure 41: Res-analogs, TIMBD and HPIMBD inhibit nuclear translocation of β-catenin in breast cancer cells, MDA-MB-231. (A) Representative Western blot showing decreased expression of β-catenin protein levels in the nuclear fractions from MDA-MB-231 cells treated with 66 μM of Res and 21 μM of TIMBD (IC$_{50}$ values of these compounds for MDA-MB-231 cells) for 6 hour; and (B) Representative Western blot showing decreased expression of β-catenin protein levels in the nuclear fractions from MDA-MB-231 cells treated with 66 μM of Res and 29 μM of HPIMBD (IC$_{50}$ values of these compounds for MDA-MB-231 cells) for 6 hour.

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5.2.5. β-catenin is negative regulator of E-cadherin.

We investigated whether silencing of β-catenin has any effect on the expression of E-cadherin gene in MDA-MB-231 cells. An increase in E-cadherin protein expression was observed after silencing of β-catenin in MDA-MB-231 cells using RNA-interference technique (Figure 42 A & 42 B).
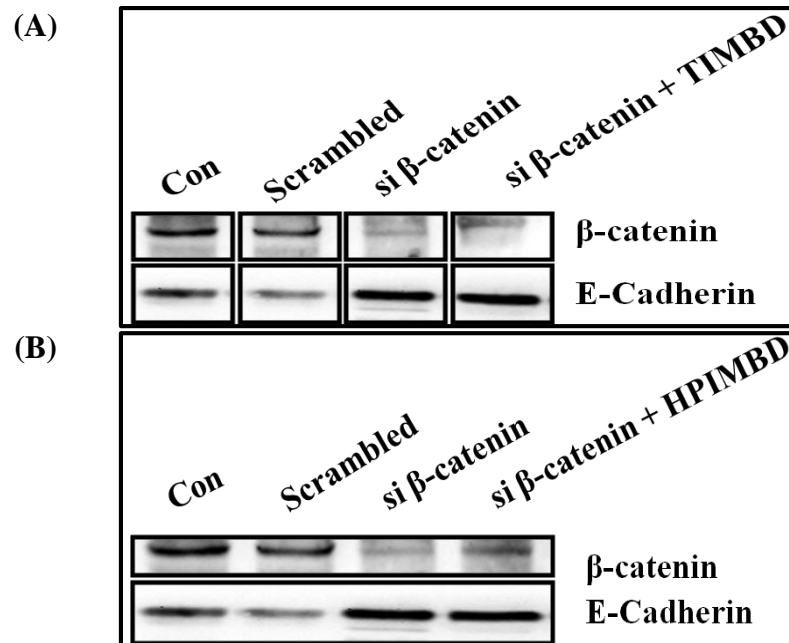
Figure 42: β-catenin is negative regulator of E-Cadherin. (A) MDA-MB-231 cells were transfected with 15 nmol/l of scrambled siRNA or si β-catenin with or without TIMBD for 48 hour, and western blot analysis was carried out using β-catenin antibody. The same membrane was reprobed with E-Cadherin antibody; (B) MDA-MB-231 cells were transfected with 15 nmol/l of scrambled siRNA or si β-catenin with or without HPIMBD for 48 hour, and western blot analysis was carried out using β-catenin antibody. The same membrane was reprobed with E-Cadherin antibody.

5.2.6. Res-analogs prevent breast cancer cell migration *in vitro.*

Considering the ability of our novel Res-analogs in down-regulation of EMT-specific molecular markers in breast cancer cells, we next evaluated the ability of these compounds in controlling migration of these aggressive metastatic breast cancer cells by wound healing assay. MDA-MB-231 cells were grown to confluence in six-well culture plates and then treated with 50 µM of Res, TIMBD or HPIMBD. Scratches were made with a 20 µl pipette tip. It took 24 hour for the wounds to completely close up in untreated cells, suggesting that these cells are very aggressive. Resveratrol treatment inhibited approximately 30% cell migration at 24 hour time point where wound closure was evaluated. Whereas TIMBD or HPIMBD inhibited approximately 50–60% cell migration after 24 hour compared to the control cells which had almost completely closed the wounds (Figure 43 & Figure 44).
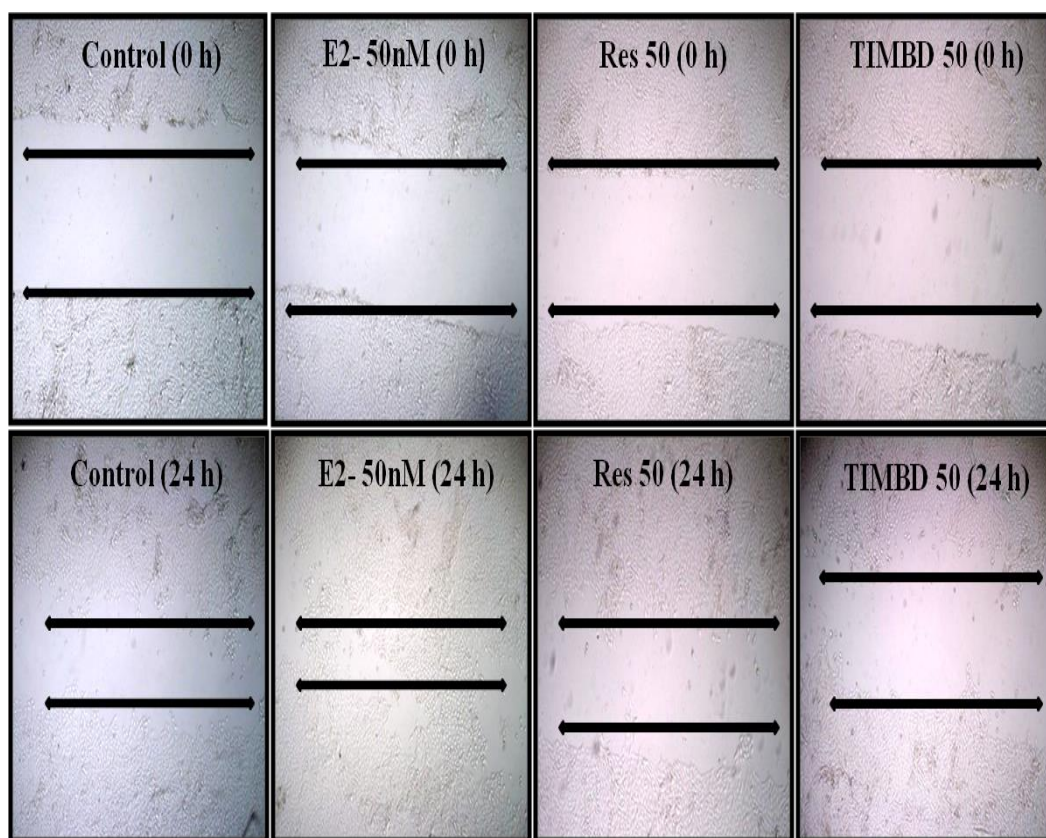
Figure 43: Res-analog TIMBD inhibits migration of aggressive breast cancer cells, MDA-MB-231 *in vitro* shown by wound-healing assay. MDA-MB-231 cells were treated with 50 nM E2, 50 μM Res or 50 μM TIMBD for 24 hour and wound-healing assays were performed as described in the Materials and methods.
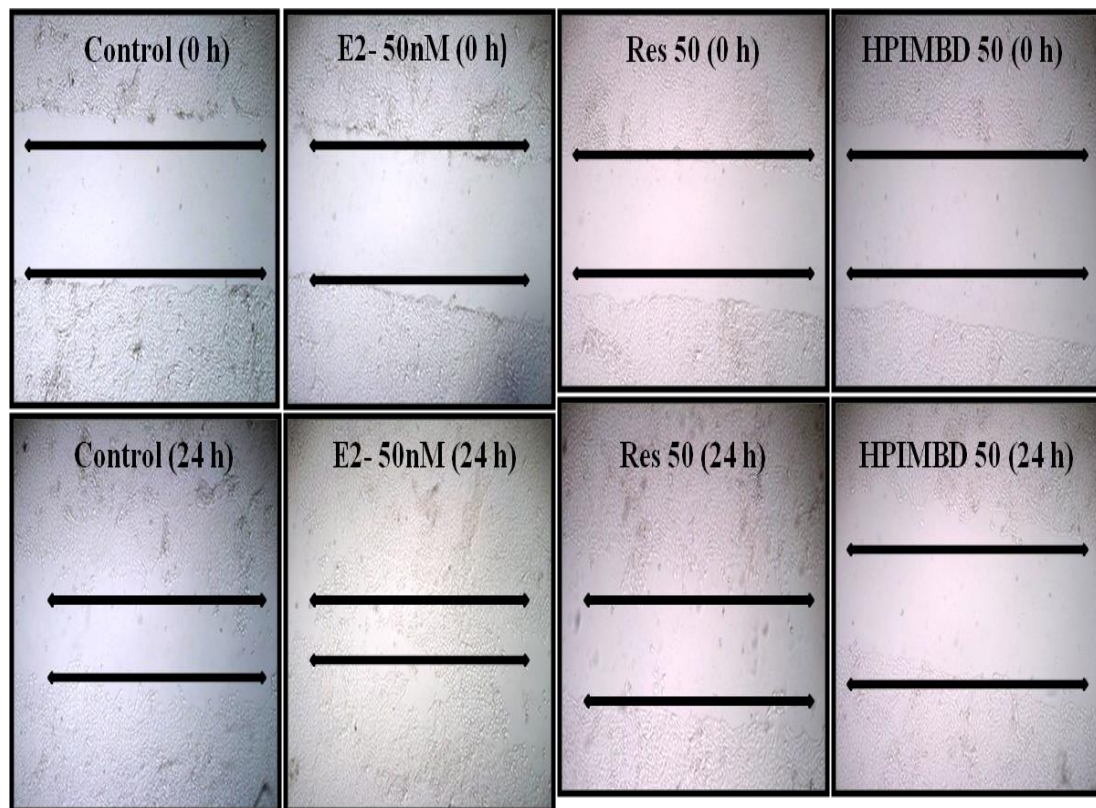
Figure 44: Res-analog HPIMBD inhibits migration of aggressive breast cancer cells, MDA-MB-231 *in vitro* shown by wound-healing assay. MDA-MB-231 cells were treated with 50 nM E2, 50 μM Res or 50 μM HPIMBD for 24 hour and wound-healing assays were performed as described in the Materials and methods.

5.3. Discussion

It has been studied recently that Res-analog bearing 3,4-dihydroxy substitution (195) is potent apoptotic agent against leukemia. It has also been reported that (196) that trihydroxyl Res-analog shows COX-2 inhibition at nanomolar range. According to some reports, Res-analog with (197) 3,4-hydroxy substitution in 'A' ring results in significant apoptotic ability of Res. Based on these observations, in order to enhance cytotoxic ability of Res towards breast cancer cells, our collaborative team have synthesized some novel analogs of resveratrol (133) where 3,4-dihydroxy substitution on the A ring of Res is kept undisturbed while changing the substituents groups at C-4 position on the B ring and introduction of Aza linkage in the conjugated system. In the present study, we hypothesized that our novel aza-Res analogs; TIMBD and HPIMBD can function as effectively as or better than Res as anti-metastatic agents against aggressive and invasive phenotypes of breast cancer by prevention of the process of EMT. The results from this current study have demonstrated the potency of Res-analogs in down regulation of mesenchymal molecular markers along with restoration of epithelial markers in human metastatic breast cancer MDA-MB-231 cells. Our findings show that TIMBD and HPIMBD are better than Res in restoring epithelial marker E-cadherin expression. The mechanism of up-regulation of E-cadherin by Res-analogs involves suppression of snail, slug and Zeb1 expressions (Figure 38). In order to dissect out the signaling mechanism involved in E-cadherin down regulation, we aimed to examine β-catenin pathway, which is frequently associated with negatively controlling the expression of E-cadherin (198). β-Catenin plays very important role in cell-cell adhesion by interacting with the cytoplasmic domain of E-cadherin and activation of this protein leads to up-regulation of

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canonical Wnt/β-catenin pathway (199, 200). Overexpression and mutations of β-catenin are associated with various human cancers including metastatic breast cancer (201). Since we found increased E-cadherin expression by Res-analogs, we hypothesized that these analogs could prevent the EMT program by targeting Wnt/β-catenin signaling. The results from this study have shown that our novel Res-analogs TIMBD and HPIMBD down-regulate the expression of β-catenin (Figure 39 B & 39 C). Furthermore, these analogs have been shown to regulate cellular distribution of β-catenin in terms of restricting its nuclear migration (Figure 41 A & 41 B). In order to find out if β-catenin could regulate the expression of E-cadherin, we knocked down β-catenin in MDA-MB-231 cells by β-catenin-specific siRNA, and confirmed that β-catenin negatively regulates E-cadherin expression (Figure 42 A & 42 B). These results are consistent with our findings that our novel analogs up-regulate E-cadherin expression with concomitant down-regulation of β-catenin expression in MDA-MB-231 cells. Wnt proteins are known to play important role in metastatic cancers (202). Wnt proteins bind to cell surface receptors, ultimately resulting in the translocation of β-catenin to the nucleus. We examined the effects of our novel Res-analogs on the expression of different wnt genes like wnt-7b, implicated to drive EMT, and wnt-5a which is known as an epithelial marker as well as tumor suppressor. Results from our studies have shown that Res-analogs down-regulate wnt-7b mRNA expression levels (Figure 39 F & 39 G) in metastatic breast cancer cells MDA-MB-231. We also found that Res-analogs significantly up-regulate epithelial specific wnt-5a in non-tumorigenic breast epithelial cells, MCF-10A (Figure 40 A & 40 B). These results support our hypothesis that TIMBD and HPIMBD prevent EMT by modulating Wnt/β-catenin pathway.

Increased expression of MMPs is also very important molecular signature of EMT which helps in the invasion and metastasis of cancer cells. It has already been reported that metastatic breast cancer cells undergoing EMT also express elevated levels of MMPs. It has also been studied that invasion and migration of breast cancer cells were prevented by blocking expression of MMPs (187). We wanted to investigate the effects of our novel Res-analogs in the expression levels of MMPs in MDA-MB-231 cells. Treatment of breast cancer cells with TIMBD and HPIMBD resulted in down-regulation of MMP3 and MMP9 but not MMP2 protein expression levels. Altogether, our study also demonstrated that several MMPs are also regulated by TIMBD and HPIMBD (Figure 37).

In conclusion, our results represent the first proof that aza-Res analogs TIMBD and HPIMBD significantly prevent the process of EMT and up-regulate molecular markers of epithelial phenotype such as E-cadherin. Furthermore, we have demonstrated the ability of these analogs to prevent metastatic breast cancer cell migration, demonstrated by wound healing assays. Our studies have shown the involvement of wnt/β catenin pathway as potential underlying mechanism of EMT inhibition by TIMBD and HPIMBD. The findings imply that TIMBD and HPIMBD decrease the invasive and metastatic properties of aggressive breast cancer cells and thus may be considered as potential therapy to prevent spread of breast cancer.

CHAPTER 6

NOVEL RESVERATROL ANALOGS TIMBD AND HPIMBD DEMONSTRATE
CYTOTOXICITY TOWARDS BREAST CANCER CELLS BY A MECHANISM
INVOLVING AN EARLY ONSET OF AUTOPHAGY

6.1. Introduction

Breast cancer is one of the most life-threatening diseases all over the world. It is very common in women of the United States and is the leading cause of cancer deaths in the United States women (1-3). The routinely used treatments of breast cancer include chemotherapy, radiation therapy, surgery etc. But standard cancer chemotherapy has major problems of adverse side effects (203-205). These side effects are caused by the non-specific targeting of the chemotherapeutic drugs, killing normal cells along with the cancer cells (203-205). Based on the hormone receptor status, breast cancer can be of major three subtypes; (I) estrogen receptor (ER) and progesterone receptor (PR) positive, that can be treated by anti-estrogen therapy (206, 207) (II) human epidermal growth factor receptor 2 positive HER-2), that can be treated by anti-HER2 antibodies that would block HER-2 function (9); and, (III) triple negative breast cancer (TNBC) cases that express neither ER, PR nor HER2 and are difficult to treat (208, 209). In the treatment of breast cancer commonly the receptors are targeted (ER, PR, HER-2) (206, 207). Triple negative breast cancer account for about 10-20% of all sub types of BC (209) Triple negative breast cancer more frequently affects younger patients and is more prevalent in African American women (210). Women with TNBC are known to have poor prognosis

than women with other BC subtypes (209). It has been reported that less than 30% of women with metastatic TNBC survive more than 5 years (211). Molecular mechanisms for the etiology, progression and pathobiology of TNBCs are not clear and this subtype is clinically characterized as more aggressive and less responsive to standard treatment (211). Significantly less progress has been made to date to effectively target TNBCs. Hence, there is a critical need to develop newer drugs with higher potency towards inhibiting the growth of TNBCs. Additionally, although a number of drugs have been developed to treat breast cancer, frequent toxicity is associated with existing drugs to normal cells (205). Therefore, it is equally critical to develop newer drugs that not only have higher potency to inhibit the growth of TNBCs but also have with minimal or no toxicity towards normal cells.

In order to overcome the limitations of conventional chemotherapeutic drugs and to develop successful agents targeting TNBC and other types breast cancers, pharmacologically active resveratrol analogs have been synthesized in such a way that they have better anticancer efficacy and specificity than that of the parent compound, Res and they have higher potential to kill breast cancer cells.

Our newly synthesized Res-analog compounds TIMBD and HPIMBD (133), have effectively inhibited the growth of both TNBCs and non-TNBCs with no/minimal toxicity towards normal cells. Our results suggest that the mechanism responsible for higher potency of TIMBD and HPIMBD towards breast cancer cells is through a relatively earlier onset of autophagy via a Notch 2 signaling pathway.

In this current work we have demonstrated that the mechanism of cytotoxic effects of TIMBD and HPIMBD towards breast cancer cells involves an early onset of

autophagy. In our preliminary studies we have found that the IC_{50} values of TIMBD and HPIMBD are of <5 μM for TNBC cell line MDA-MB-468 (derived from African American breast cancer patient). Thus, development of TIMBD and HPIMBD as novel therapeutic compounds for breast cancer inhibition will also have the advantage of inhibiting the most aggressive type of breast cancer.

6.2. Results

6.2.1. Res-analog TIMBD and HPIMBD show higher potency for inhibiting the proliferation of TNBC cells compared to non-TNBC cells.

We have tested newly synthesized Res-analogs for their potential to inhibit proliferation of breast cancer cells using MTT assay. In our initial screening, we used 5 breast cancer cell lines (MCF-7, T47D, MDA-MB-468, MDA-MB-231 and BT20) and 3 non-neoplastic breast epithelial cell lines (MCF-10A, MCF-10F and HMEC). MCF-7 and T47D are non-TNBC whereas MDA-MB-231, MDA-MB-468 & BT20 are TNBC cell lines. TIMBD and HPIMBD show better potency ($p<0.05$) than Res in inhibiting the growth of all breast cancer cell lines that we tested (Table 2). Importantly, TIMBD and HPIMBD show higher potency for inhibiting the proliferation of TNBC cell lines compared to non-TNBC cell lines that we tested ($p<0.05$, Table 2). These two analogs have no detectable effect on the growth of any of the 3 non-neoplastic breast epithelial cell lines that we tested upto a dose of 50 μM (table 2). HPIMBD is ~9 times and TIMBD is ~5 inhibitory for TNBC cell line MDA-MB-468 compared with non-TNBC cell lines MCF-7 or T47D as suggested by their IC_{50} values. Thus, TIMBD or HPIMBD not only inhibit breast cancer cell proliferation, they have a preference towards TNBC cells.

6.2.2. TIMBD and HPIMBD show anti-clonogenic activity in breast cancer cells better than Res

About 500 viable MDA-MB-231 cells were seeded in six-well plates and allowed to grow for 24 hour in phenol red-free complete media. The cells were then treated with or without increasing dosages of Res, TIMBD or HPIMBD (5 μl, 10 μl and 25 μl) for 72 hour, washed in PBS and incubated for an additional 8 days in complete medium. The colonies obtained were washed with PBS and fixed in 10% formalin for 10min and again washed twice with PBS followed by staining with crystal violet (0.1% w/v solution in 10% ethanol). The colonies were counted, photographed and compared with respective untreated cells (Figure 45). Each treatment was done in triplicate.
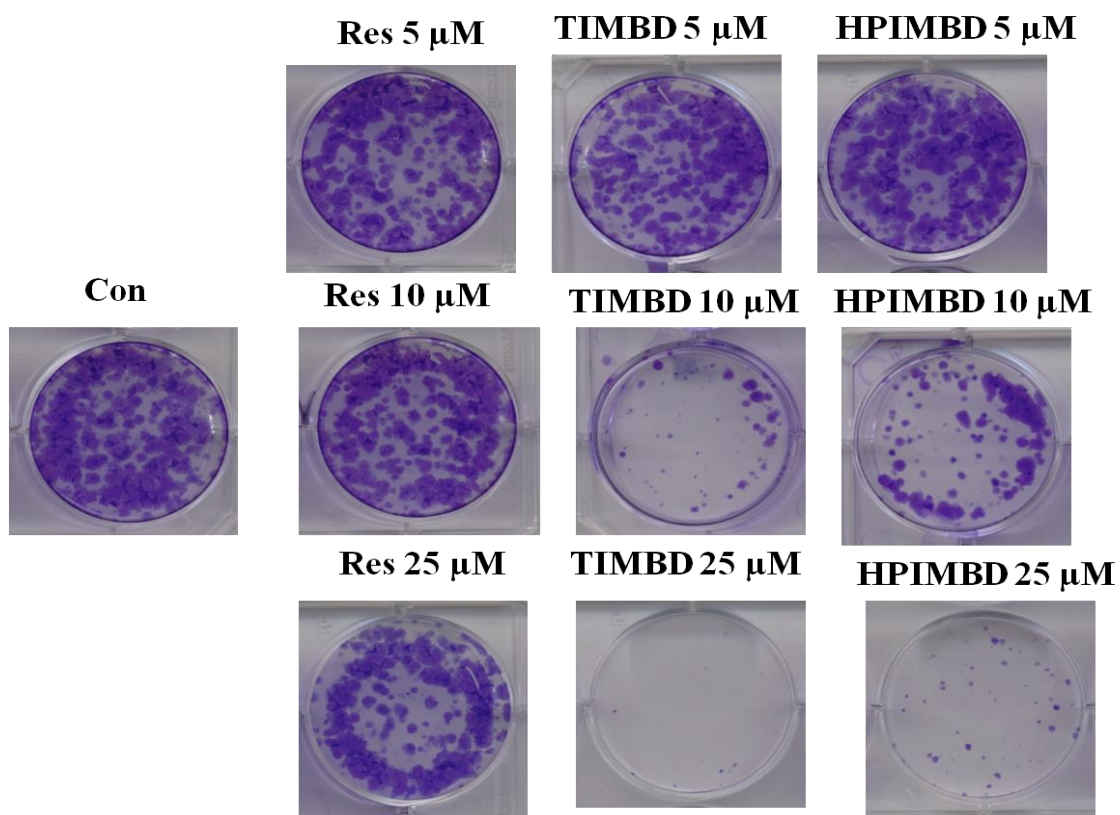
Figure 45: Comparison of Res, TIMBD and HPIMBD for anti-clonogenic activity in breast cancer cells, MDA-MB-231 shown by colony formation assay. MDA-MB-231 cells were treated with increasing concentrations of Res, TIMBD or HPIMBD for 24 hour and colony formation assays were performed as described in the Materials and methods.

6.2.3. TIMBD and HPIMBD inhibit mammosphere formation in breast cancer cells better than Res

Mammosphere formation assay was carried out as described in materials and method section. Briefly, 5000 viable T47D cells were seeded in ultra-low attachment plates. After 24 hour, cells were treated with 50 µM of Res, HPIMBD and TIMBD and were grown specific media. After 7-8 days of incubation, mammospheres were viewed under the microscope and photographed. Three replicate wells from a 24-well plate were used for each experimental condition. 50 nM of E2 induced mammosphere formation; 50 µM of TIMBD and HPIMBD significantly inhibited formation of mammospheres; 50 µM of Res could not inhibit mammosphere formation (Figure 46).
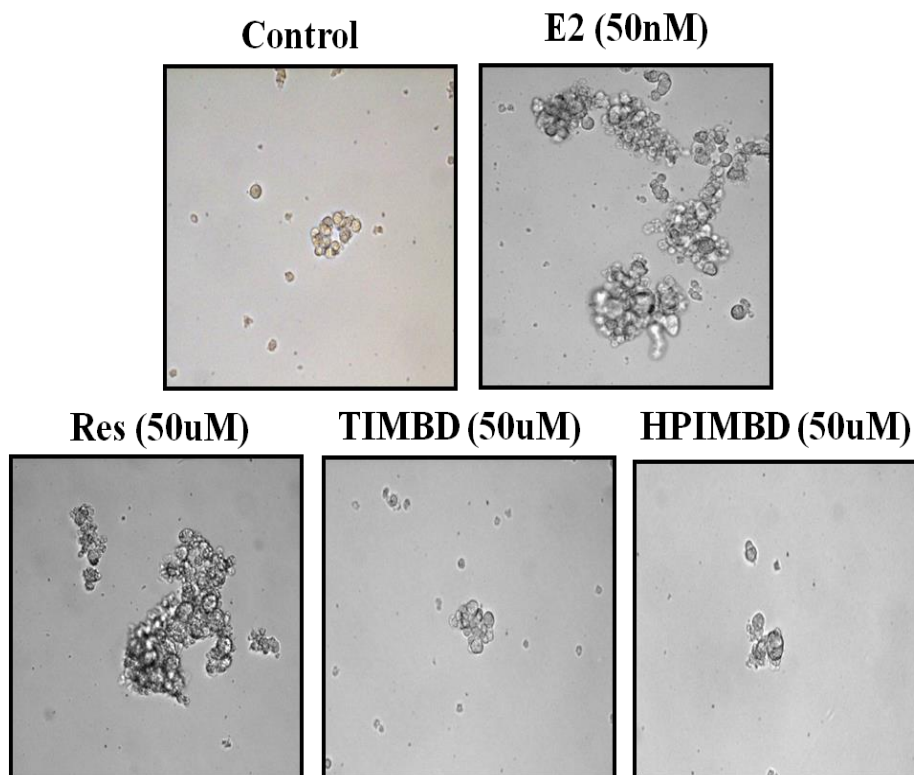
Figure 46: Comparison of Res, TIMBD and HPIMBD for anti-tumorigenic activity in breast cancer cells, T47D as shown by mammosphere formation assay. T47D cells were treated with 50 µM concentrations of Res, TIMBD or HPIMBD for 24 hour and mammosphere / spheroid formation assays were performed as described in the Materials and methods.

6.2.4. TIMBD and HPIMBD show inhibition of growth of breast cancer cells by inducing autophagy.

Our present studies indicate that TIMBD and HPIMBD increase Beclin-1 and LC3II protein expression levels in TNBC cell lines MDA-MB-231, MDA-MB-468 and non-TNBC cell line T47D (Figure 48), which support autophagy-mediated inhibition of cell growth. MDA-MB-231 cells exposed to TIMBD and HPIMBD displayed double membrane cytoplasmic structures known as autophagosomes stained with the FITC-fluorescent MAP-LC3 antibody (Figure 47 A & 47 B). Beclin-1 expression is reported to be low in breast cancers and its over expression is reported to inhibit breast cancer. Thus, our current studies indicating increased expression of Beclin-1 by TIMBD (Figure 48 A) and HPIMBD (Figure 48 B) show clinical relevance of the potential of these agents in inhibition of breast tumors. The mechanism of inhibition by TIMBD or HPIMBD is potentially different from the documented apoptotic mechanism of inhibition by Res. Our present studies show activation of caspase 3/7 by Res whereas TIMBD or HPIMBD show inhibition of caspase 3/7 activity, a known marker of apoptosis (Table 3). TIMBD or HPIMBD increase protein expression levels of Beclin-1 and LC3II, known markers of autophagy whereas Res has no detectable effect on protein expression levels of these proteins (Figure 48 A & 48 B).
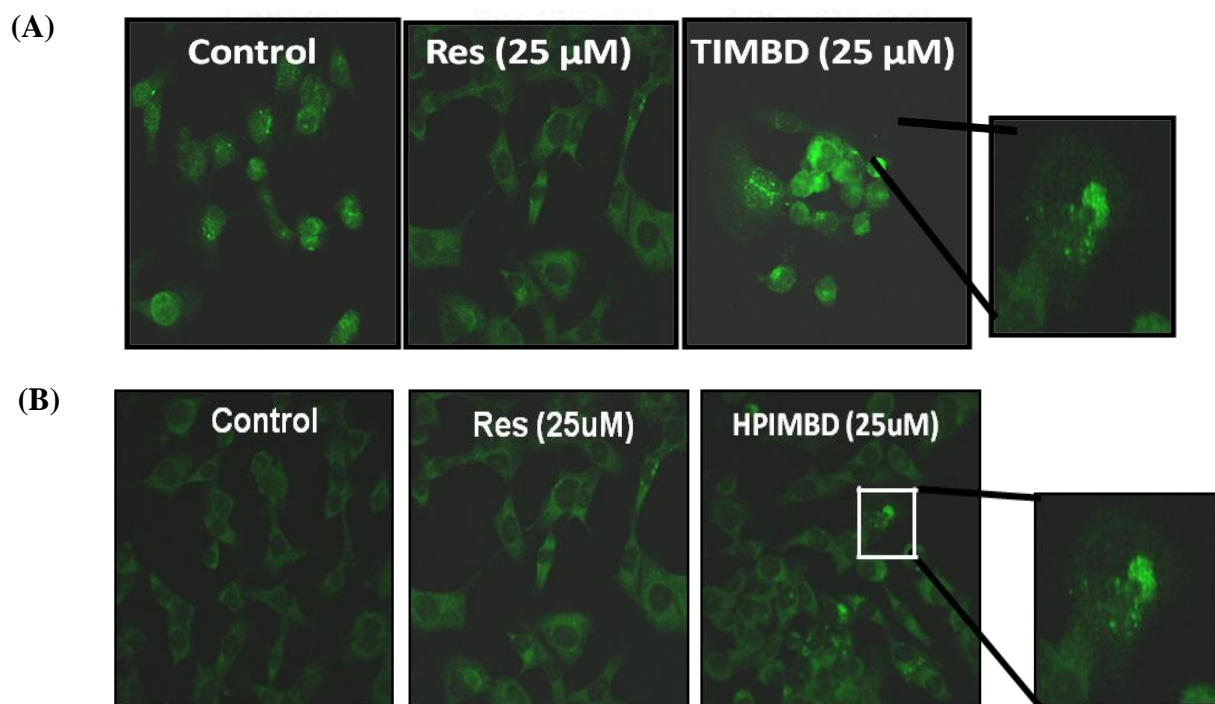
Figure 47: Representative LC3 immunocytochemistry pictures showing formation of autophagosomes by Res-analogs in MDA-MB-231 cells. (A) Cells were treated with TIMBD (25 µM) for 12 hour, compared with that of vehicle-treated control or Res-treated cells. Right, enlarged image showing an autophagosome formed in TIMBD-treated cell; (B) Cells were treated with HPIMBD (25 µM) for 12 hour, compared with that of vehicle-treated control or Res-treated cells. Right, enlarged image showing an autophagosome formed in HPIMBD-treated cell.

Table 3: Caspase 3/7 activity in MDA-MB-231 and T47D cells treated with Res, TIMBD or HPIMBD (50μM) for 24 hour. Resveratrol showed significant increase in caspase 3/7 activity in these breast cancer cells suggesting apoptosis-mediated cell death. In contrast, TIMBD or HPIMBD showed significant decrease in caspase 3/7 activity which suggests these Res-analogs do not mediate apoptotic cell death like that of Res. '*' indicates p value ≤ 0.05 compared with respective controls.

TREATMENT	CASPASE 3/7 ACTIVITY (Fold change VS. control) MDA-MB-231	CASPASE 3/7 ACTIVITY (Fold change VS. control) T47D
Control	1	1
Res	1.76 *	1.81 *
TIMBD	0.69 *	0.62 *
HPIMBD	0.58 *	0.55 *

(A)

Con   Res   TIMBD

MDA-MB-231
- Beclin-1
- ← LC3-I
- ← LC3-II
- α-Tubulin

Con   Res   TIMBD

MDA-MB-468
- Beclin-1
- ← LC3-I
- ← LC3-II
- α-Tubulin

Con   Res   TIMBD

T47D
- Beclin-1
- ← LC3-I
- ← LC3-II
- α-Tubulin

(B)

Con   Res   HPIMBD

MDA-MB-231
- Beclin-1
- ← LC3-I
- ← LC3-II
- α-Tubulin

Con   Res   HPIMBD

MDA-MB-468
- Beclin-1
- ← LC3-I
- ← LC3-II
- α-Tubulin

Con   Res   HPIMBD

T47D
- Beclin-1
- ← LC3-I
- ← LC3-II
- α-Tubulin
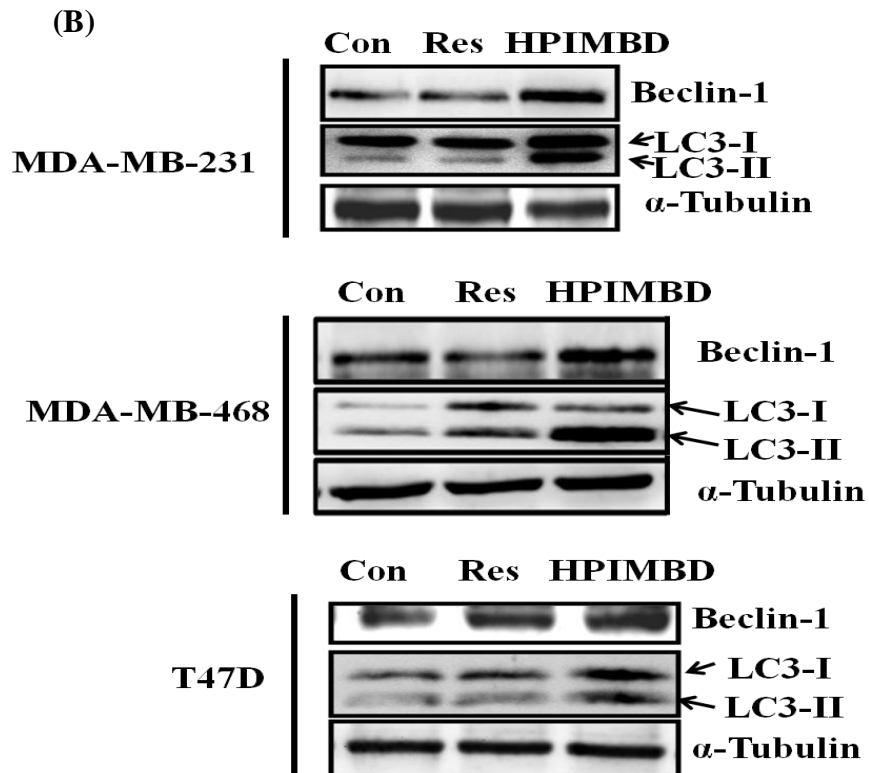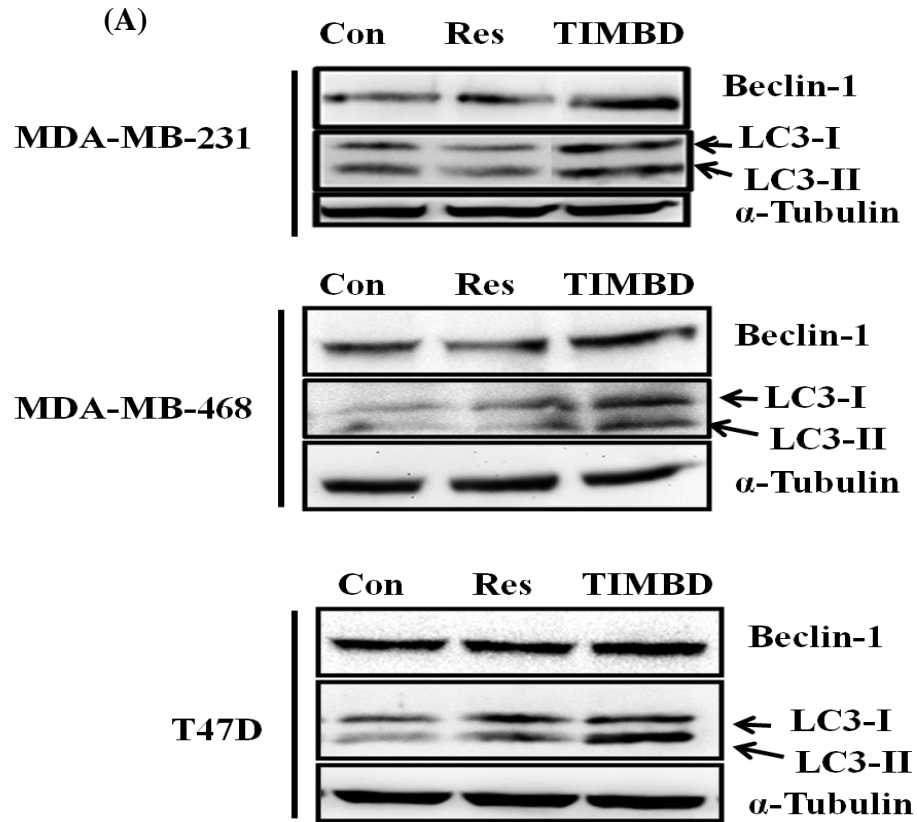
Figure 48: Res-analogs, TIMBD and HPIMBD shows induction of autophagy in different breast cancer cells. (A) Representative Western blots for Beclin-1 and LC3 in MDA-MB-231, MDA-MB-468 and T47D cells treated with Res or TIMBD for 12, 6 and 24 hour, respectively; and (B) Representative Western blots for Beclin-1 and LC3 in MDA-MB-231, MDA-MB-468 and T47D cells treated with Res or HPIMBD for 12, 6 and 24 hour, respectively.

 *p<0.05 compared to vehicle-treated cells.

6.2.5. TIMBD or HPIMBD induce autophagy via Notch 2 pathway.

Our present results demonstrate that TIMBD (Figure 50) and HPIMBD (Figure 51) increase Notch 2 protein levels with induction at an earlier time point in TNBC cell line MDA-MB-231 (6 hour) compared to non-TNBC cell line T47D (12 hour). Concomitant with an increase in Notch 2 protein levels, there is a corresponding increase in Beclin-1 protein levels (Figure 50 & Figure 51). Protein expression levels of Beclin-1 and Notch 2 remain high in TNBC cell line MDA-MB-231 for upto 12 hours. Silencing of Notch 2 using siRNA to Notch 2, results in decreased expression of Beclin-1 (Figure 49). These results suggest that Notch 2 may play an important role in TIMBD and HPIMBD-mediated induction of autophagy.
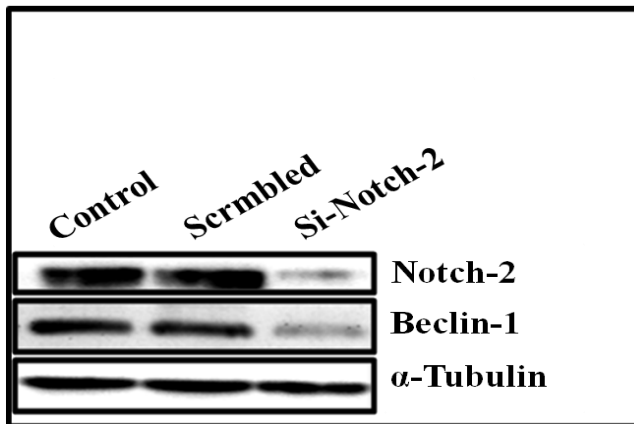
Figure 49: Notch 2 positively controls the expression of autophagy-related protein Beclin-1. Representative western blot showing decreased Beclin-1 protein expression in si Notch 2 transfected MCF-10A cells.
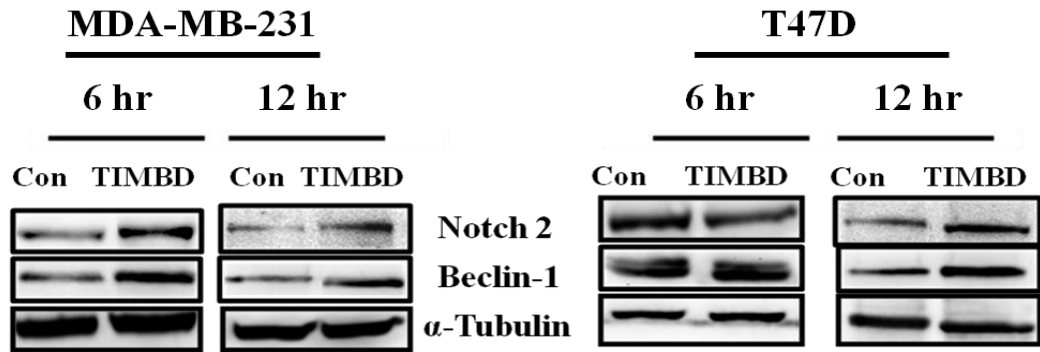
Figure 50: Res-analog TIMBD induces autophagy via Notch 2 pathway. Representative western blots showing Notch 2 and Beclin-1 protein expressions in MDA-MB-231 and T47D cells, following TIMBD (50 µM) treatment.
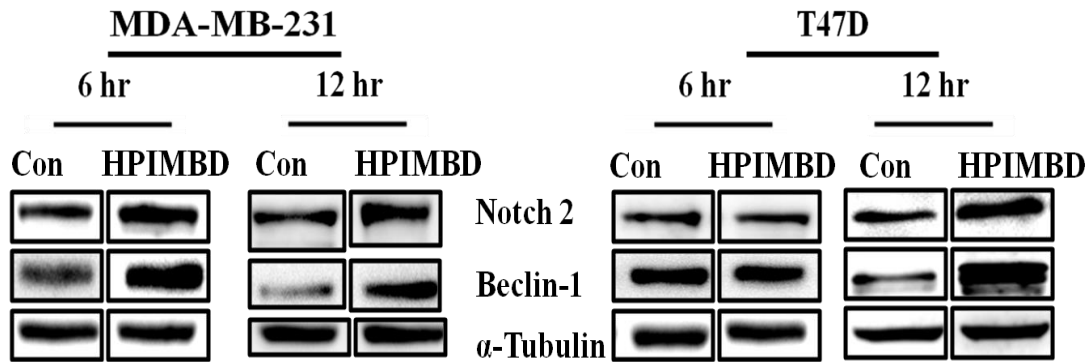
Figure 51: Res-analog HPIMBD induce autophagy via Notch 2 pathway. Representative western blots showing Notch 2 and Beclin-1 protein expressions in MDA-MB-231 and T47D cells, following HPIMBD (50 µM) treatment.

6.3. Discussion

Triple negative breast cancer (TNBC) comprises of 15-20% of all breast cancers and is the most aggressive and invasive subtype of breast cancer. In general, for treatment of breast cancers, hormonal receptor status is essential to be determined in order to target cancer cells. Treatment for breast cancer largely depends on the presence of ER, PR or HER-2 receptors in the cancer cells. But TNBC is that sub type of breast cancer that lacks positive hormone receptor status and that is why chemotherapeutic treatment targeting hormonal receptors and molecular markers all are ineffective to treat TNBC. Although Chemotherapy is still the major strategy for treatment of TNBC, reoccurrence of the disease is a challenging drawback. Therefore, it is very important to develop novel drugs with higher potency towards inhibiting the growth of TNBCs. The pharmacological agents that have been developed for treatment of breast cancer are found to exert toxicity to normal cells (203-205). Therefore, it is equally important to develop novel therapeutic agents that should have specificity towards inhibiting growth of TNBCs having minimal or no toxicity towards normal cells.

Nature has endowed us with a variety of non-synthetic natural compounds with unique medicinal values. One of the hugely studied naturally occurring plant derived phyto-estrogen is namely resveratrol (Res), well studied for its medicinal properties. It is found in various vegetables, nuts, fruits including grapes and red wine. Res have been well explored for its plethora of medicinal properties in treatment of cancer and a wide range of other diseases (90-92). Resveratrol mediates its anticancer activity by inducing apoptosis in cancer cells via modulating various signal transduction pathways and targeting different transcription factors responsible for uncontrolled growth of cancer

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cells (115-119). Not only limited to *in vitro* studies, Res has also been successfully shown to reduce growth of primary tumor xenografts in animal model (120).

We have recently synthesized some novel resveratrol analogs by maintaining 3,4-dihydroxy substitution on the A ring while varying the substituents at C-4 position on the B ring along with inclusion of Aza functionality in the conjugated system (133). The analogs synthesized were named as TIMBD and HPIMBD (133), have extensively studied for their anti proliferative properties on breast cancer cells. TIMBD and HPIMBD have shown to inhibit breast cancer cell proliferation effectively and successfully with no or least toxicity towards normal breast epithelial cells.

In this current work we have shown the potency of TIMBD and HPIMBD in inhibiting the proliferation of breast cancer cells with a preference towards TNBC cells (Table 2). We found the IC_{50} of TIMBD and HPIMBD of <5 µM in the TNBC cell line MDA-MB-468 (Table 2). It is reported that IC_{50} value of <5 µM is well within the acceptable range for evaluation of a drug for efficacy and safety in a clinical setting (139-140). Thus, in this current study we aimed at developing TIMBD and HPIMBD as novel therapeutic compounds for treatment of breast cancer with minimal or no toxicity towards normal breast epithelial cells (Table 2). To further delineate the mechanism responsible for higher potency of TIMBD and HPIMBD towards breast cancer cells, we found is through a relatively earlier onset of autophagy (Figure 48) via a Notch 2 signaling pathway (Figure 49-51).

There are two major types of cell death processes cumulatively called as Programmed cell death, apoptosis (Type-I) and autophagy (Type-II) (212, 213). Autophagy, is defined as a cellular process that mediates degradation of cellular

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components through formation of autolysosomes which are formed by fusion of autophagosomes with lysosomal vesicles (214). Autophagy is reported to play important role in cancer and may be defective in tumour cells (215). It is evident from literature that autophagic cell death is an alternative pathway to target cancer cells and chemotherapeutic agents often induce this pathway where apoptotic cascades are inactive (216). According to these scientific evidences it might be said that molecular players of autophagy are very important to prevent the process of carcinogenesis. In the current study, we demonstrated that the novel resveratrol analogs TIMBD and HPIMBD induce the process of autophagic cell death in breast cancer cell lines (Figures 47 & Figure 48). We have used human immortalized estrogen receptor positive breast cancer cell line, T47D and TNBC cell lines MDA-MB-231 and MDA-MB-468 for our studies. MDA-MB-231 cells exposed to TIMBD and HPIMBD displayed double membrane cytoplasmic structures known as autophagosomes staining with the FITC-fluorescent MAP-LC3 antibody (Figure 47 A & 47 B). To confirm autophagic protein activation by TIMBD and HPIMBD, we studied induction of autophagy specific protein Beclin 1 (217) and conversion of the soluble form of LC3 which is found on the autophagosome membrane (LC3-I) to lipidated and autophagosome-associated form (LC3-II) by western blotting (Fig. 3B). Beclin 1 protein expression is reported to be lower in breast cancer cells and its poor expression is correlated with development of breast cancer (217). In our current study we have demonstrated that TIMBD and HPIMBD induce Beclin 1 in TNBC as well as non-TNBCs, with an early onset in the later cell lines (Figure 48 A & 48 B). To make sure that our novel Res-analog induces cancer cell death by autophagy and not by type-I cell death apoptosis, we performed caspase3/7 activity assay and TIMBD and HPIMBD

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showed down regulation of caspase3/7 activity (Table 3). These results clearly suggest that TIMBD and HPIMBD kill cancer cells by inducing autophagy and not by apoptosis like its parent compound, Res (115).

Notch signaling is a very important cell signaling pathway involved in carcinogenesis development and is considered as tumor-suppressor (218). There are four isoforms of Notch heterodimeric transmembrane receptors (Notch 1 through Notch 4) expressed in Humans (219). It is known that the different Notch receptors display diversity in their functions (218). In breast cancer, there is strong evidence that Notch 1 and Notch 4 are oncogenic, whereas Notch 2 may play a tumor-suppressive role (218, 220). Biologically, there is evidence that Notch 2 has opposite effects compared with Notch 1 in human breast cancer (220).

In this study, we show that Notch 2 signaling is involved in potentiating TIMBD and HPIMBD for their autophagic activities towards breast cancer cells (Figure 50 & Figure 51). This study may be very important to discover novel pharmacological interventions for treatment of breast cancer including very aggressive TNBCs.

CHAPTER 7

ANIMAL STUDIES

7.1. HPIMBD inhibits tumor development *in vivo* in an orthotopic mouse xenograft model.

We have done some preliminary experiments with laboratory animals to study the efficacy of HPIMBD in inhibition of tumor growth. As a model system for our studies we have used an orthotopic nude mouse xenograft model of breast cancer. In our pilot studies with animals, nude mice (n=2) were injected with 1×10^6 MDA-MB-231 breast cancer cells in the mammary fat pad. All mice were given free access to phytoestrogen-free food and water. Animals were injected with HPIMBD (in DMSO) intraperitonially at a dose of 50mg/kg body weight, two days before injection with MDA-MB-231 cells. We repeated this dose on every 2nd day, for 6 weeks. In our experience, tumors are usually generated within 2-3 weeks of cells' injection into nude mice. According to our observations, no visible tumors developed in mice injected with MDA-MB-231 cells and HPIMBD, while tumors developed in control nude mice injected with MDA-MB-231 cells and DMSO (Figure 52). These results imply that HPIMBD has the potential to inhibit breast tumor growth in *in vivo* systems. Nevertheless, these data are preliminary and need to be confirmed using more mice based on power analyses (n=15) to provide statistically supportive results.

We performed the same experiments using xenograft with estrogen receptor (ER) positive cells T47D. In our preliminary feasibility experiments to check the efficacy of

HPIMBD in inhibition of E2-dependent tumor growth, nude mice (n=2) were implanted with E2 two days before injection with $1x10^6$ T47D cells in mammary fat pad. On the day of implantation of E2, mice were also injected mice subcutaneously with HPIMBD dissolved in DMSO at 50mg/kg body weight. This dose was repeated on every alternate day for 4 weeks. We did not observe any visible tumors developed in mice injected with T47D and HPIMBD while tumors developed in control mice treated with T47D cells alone (Figure 53). Our results imply that HPIMBD has the potential to inhibit E2-dependent tumor growth.
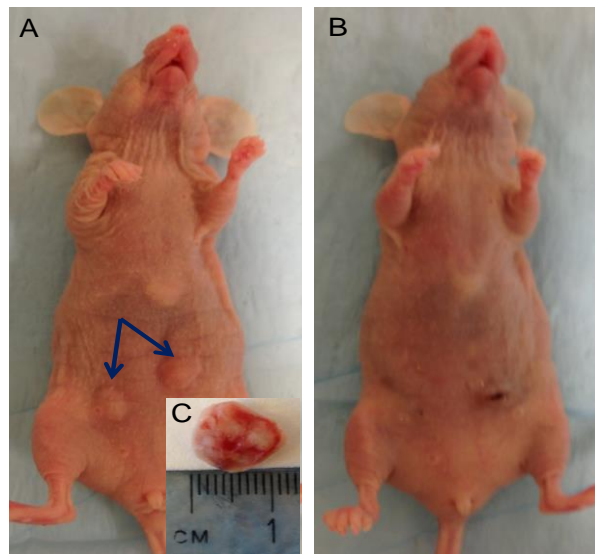
Figure 52: HPIMBD inhibits tumor development *in vivo* in an orthotopic mouse xenograft model. (A) Development of visible tumors near nipples of a nude mouse injected with MDA-MB-231 cells; and (B) No visible tumors in a nude mouse treated simultaneously with HPIMBD. C= tumor size excised from MDA-MB-231 injected mouse.
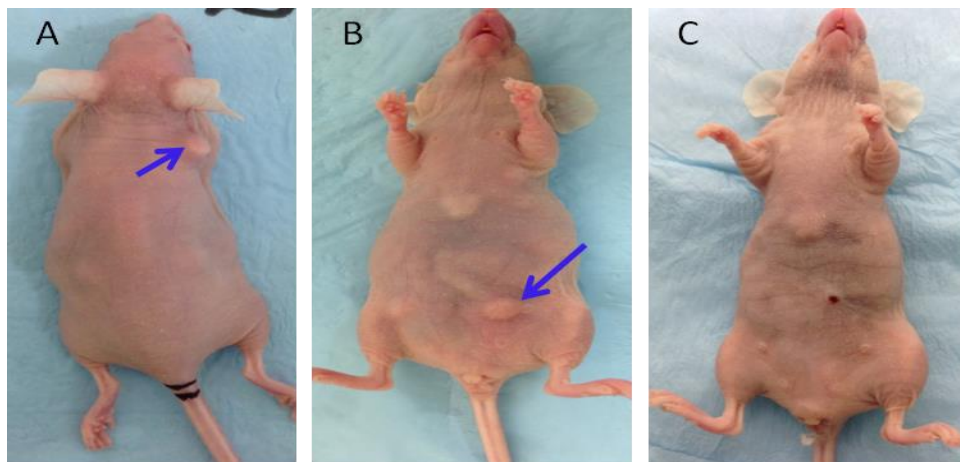
Figure 53: HPIMBD inhibits tumor development *in vivo* in an orthotopic mouse xenograft model. (A) E2-pellet implanted; (B) Development of visible tumor near nipples of a nude mouse injected with T47D cells; and (C) No visible tumors in a nude mouse treated simultaneously with HPIMBD.

SUMMERY & FUTURE PERSPECTIVE

Cancers can be categorized as preventable diseases as onset of this disease is largely connected to lifestyle and daily food habits. Cancer occurrence thus can be significantly reduced by maintaining a healthy diet. It has largely been suggested that consumption of plant-based diets which contain large amount of antioxidants, can be protective against carcinogenic insult. It has also been suggested that by reducing intake of red meats, fried, high salt and high sugar-rich foods cancer can be avoided. It is thus very clear that regular consumption of various plant-derived chemicals or phytochemicals found in a variety of fruits, nuts, vegetables and grains will significantly reduce the risk of cancer occurrence. Long-term consumption of plant-derived foods rich in antioxidants like VC and Res is well known to exert significant health benefits. In contrast with conventional synthetic cancer drugs, natural chemopreventive compounds have been found to be more beneficial in specifically inhibiting cancer cell growth with low cytotoxic effect to the normal cells. Although modern chemotherapeutic drugs have resulted in significant survival of breast cancer patients but these drugs have limitations in terms of significant side effects and development of resistance. For example, anthracyclines, cyclophosphamide, taxanes, tamoxifen etc which are routinely used in the drug regimen of breast cancer patients have significant adverse side effects due to their toxic effects on normal cells. Not only this, but these chemotherapeutic agents are also associated with the development of drug resistance. All these reasons result in withdrawal of chemotherapeutic treatments in >20% of women.

According to the epidemiological studies, the rates of breast cancer incidence are lower among Asians which is suggestive of the fact that Asian women consume food which help them to prevent breast cancer onset. Studies from various *in vitro* experiments have shown beneficial effects of naturally occurring antioxidants like VC or Res in breast cancer chemoprevention. But *in vivo* studies have demonstrated the limitations of these natural phytochemicals which holds us back to use them in clinical settings. Lower efficacy is an issue with most of the phytochemicals including resveratrol which limit the achievement of their physiologically effective doses in the *in vivo* system.

It is thus very clear that in order to make natural phytochemicals effective in *in vivo* systems and use them in clinical settings chemical modifications are needed to make these natural compounds very stable as well as selective. We have synthesized Res analogs, namely TIMBD and HPIMBD to improve the efficacy of Res. We have extensively studied these analogs for their anti-proliferative properties on breast cancer cells. Our results suggest that TIMBD and HPIMBD specifically inhibit the proliferation of human breast cancer cells. Additionally, we observed that these novel analogs had no cytotoxic effects on the growth of human non-neoplastic breast epithelial cells. These results with TIMBD and HPIMBD were very promising and encouraging to develop them as potential anti breast cancer agents. We also delineated the possible molecular mechanisms of chemoprevention by these analogs.

Our aim is to develop novel res-analogs TIMBD and HPIMBD as potential therapeutic agents that can be used to selectively inhibit development of breast cancer with no or minimal toxicity towards normal cells of the patient. To fulfill our goal, we have extensively studied these two compounds for their ability to target different genes

and proteins and various signaling pathways. We have observed that TIMBD and HPIMBD are very potent to scavenge reactive oxygen species (ROS) production and provide antioxidant defense to normal breast epithelial cells by reducing ROS-induced oxidative DNA damage. When we started looking in to the possible molecular mechanism underlying the antioxidant properties of TIMBD and HPIMBD, we observed that these two analogs are very potent in inducing cellular phase-II detoxifying genes like SOD3 and NQO1. We have then demonstrated that TIMBD and HPIMBD up regulate cellular Phase-II detoxifying system by up regulation of transcription factor Nrf2, the master regular of antioxidant defense system. In addition to Nrf2, we studied other members of CNC-bZip family of proteins which are known to play important roles in antioxidant defense in mammalian cells, Nrf1 and Nrf3. We have studied Nrf1 and Nrf3 for their specific roles in hormonal breast cancer, in particular, E2-dependant breast cancer and the effects of TIMBD and HPIMBD in the regulation of these transcription factors. Results from our extensive studies have suggested that TIMBD and HPIMBD confer antioxidant defense to the normal breast epithelial cells and protect them from carcinogenic insult by modulating mRNA and protein expression levels of Nrf1, Nrf2 and Nrf3. Here, in this present study, we have presented evidence that our novel Res-analogs TIMBD and HPIMBD are very effective antioxidant agents. These analogs thus can provide defense to the normal breast epithelial cells from carcinogenic insult by reducing ROS and oxidative DNA-damage which are frequently associated with onset of carcinogenic process. Based on the results that we have obtained it can be concluded that since TIMBD and HPIMBD specifically kill human breast cancer cells without having toxicity towards normal breast epithelial cells, they could be used as promising

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chemotherapeutic agents for the treatment of breast cancer patients. In this way we may avoid the adverse side effects of otherwise used conventional chemotherapeutic agents in breast cancer. With our observations of novel res-analogs having potent antioxidant properties, we could use them for prevention of breast cancer. Whether the effects of TIMBD and HPIMBD will show promising results for prevention as well as treatment of breast cancer in the clinical settings, it is still a challenging question one could ask. It will be imperative to do animal experiment studies using significant number of model animals receiving TIMBD and HPIMBD in their daily diets, to prove these drugs efficacy as anti-tumor and chemopreventive agents. Although we have developed Nude mouse orthotopic model of breast cancer in our laboratory and used them to study tumor prevention as well as tumor inhibition by administrating TIMBD or HPIMBD intraperitonially, there were not sufficient number of animals used to make a significant conclusion. But based on our pilot animal studies we observed a significant regression of breast tumor in these after administration of TIMBD or HPIMBD. We also observed that tumor development could be prevented in these experimental animals by giving them TIMBD or HPIMBD prior to implantation of breast cancer cell xenografts. These pilot animal studies have given promising results based on which we can design animal experiments in a larger scale to provide evidence that TIMBD and HPIMBD are promising agents for treatment as well as prevention of breast cancer.

Reoccurrence and metastasis are frequently associated with breast cancer. Breast cancer metastasis can become worst; resulting in migration of the cancer cells in to the other organs of the body like lungs and bones and ultimately can cause death. Treatment for metastatic breast cancer includes chemotherapy, radiation therapy, surgery etc. At

present, Treatment of metastatic breast cancer is an active and dynamic field of research. Various novel anti-metastatic drugs are being developed to particularly treat metastatic breast cancers. In order to develop TIMBD and HPIMBD as potential anti-tumor agents, we aimed to examine the anti-metastatatic properties of these novel analogs of Res. Our results have presented the first proof evidence that TIMBD and HPIMBD can significantly prevent the molecular process of epithelial-mesenchymal transition (EMT) and breast cancer cell metastasis in *in vitro* system. Next we have dissected out the possible molecular mechanisms underlying anti-metastatic activities of TIMBD and HPIMBD in breast cancer. We have observed that these analogs can significantly down-regulate matrix metalloproteases (MMPs) which are implicated in driving cell metastasis; and by up regulating molecular markers of epithelial phenotype such as E-cadherin. Furthermore, we have demonstated the ability of these analogs to prevent metastatic breast cancer cell migration. Furthermore, our studies have shown that suppression of wnt/β catenin signaling pathway could be a potential underlying mechanism of EMT inhibition by our Res-analogs. Taken together, these findings suggest that TIMBD and HPIMBD can act as potential anti-metastatic agents and thus can be used successfully to decrease the invasive and metastatic properties of aggressive breast cancer cells.

In the 6th chapter we have discussed about the possible mechanisms by which our novel res analogs target breast cancer cells and inhibit their growth. We found TIMBD and HPIMBD induced Beclin-1 and LC3II which suggest autophagy mediated inhibition of cell growth. Beclin-1 is known to be suppressed in breast cancers and it's over expression is reported to inhibit breast cancer. Results from our studies demonstrate that Res-analogs TIMBD and HPIMBD are better than Res in inhibiting specifically breast

cancer cell growth and shows higher potency for inhibiting the growth of triple negative breast cancer cells by inducing autophagy with an earlier onset for triple negative breast cancer cells. Therefore, TIMBD and HPIMBD may be better chemotherapeutic agent than Res against breast cancer and more specifically against triple negative breast cancer cell growth.

In conclusion, it can be inferred that our newly synthesized Res-analogs TIMBD and HPIMBD have significant potential to inhibit growth of breast cancer cells without having any toxicity towards normal cells and they can be developed as potential chemotherapeutic agents for breast cancer. In future studies, we will investigate the pharmacokinetics and pharmacodynamics parameters of TIMBD and HPIMBD and test them in clinical settings. Our ultimate and long term goal is to develop TIMBD and HPIMBD as promising chemotherapeutic agents in breast cancer and to protect individuals from breast-cancer related deaths.
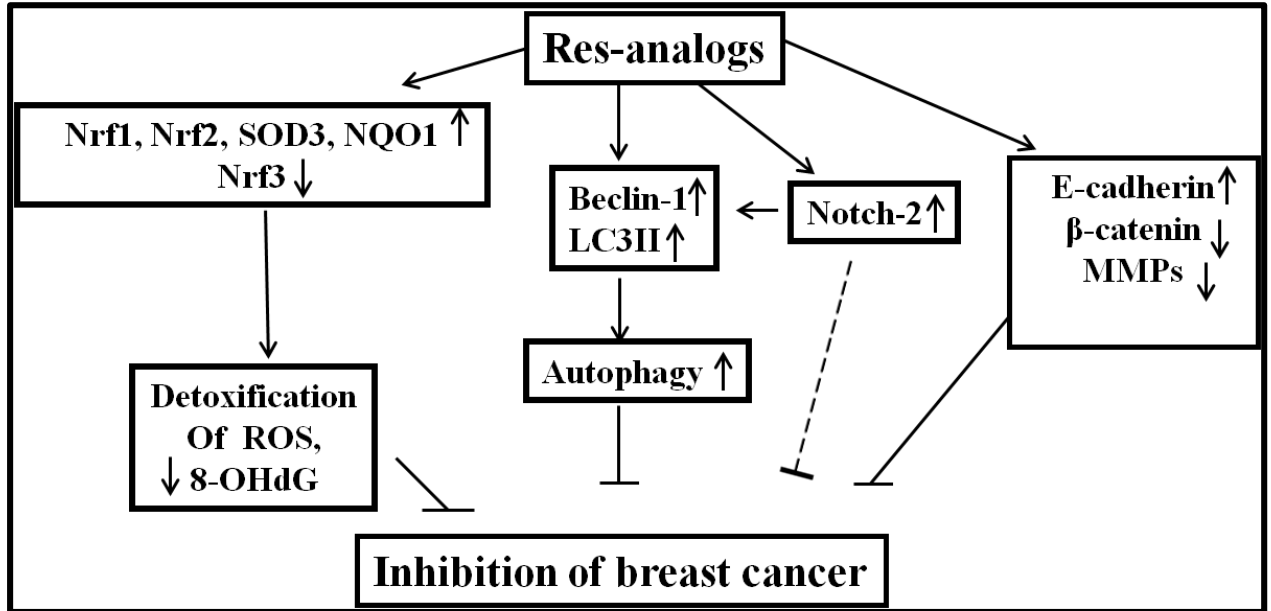
Figure 54: Resveratrol analogs: potential mechanism of chemoprevention in breast cancer

APPENDIX

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VITA

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