ANTI-BREAST CANCER ACTIVITIES OF 2α-HYDROXYURSOLIC ACID: POTENTIAL MECHANISMS OF ACTION

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XUE JIANG

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XUE JIANG, Ph. D.

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As the most common cancer diagnosed among women in the United States, breast cancer accounts for nearly one third of all cancers in women. MDA-MB-231 breast cancers are triple-negative breast cancer, which lacks an established therapeutic target. Epidemiological studies showed that increased consumption of foods with rich phytochemicals, such as whole grains, vegetables, and fruits, has been associated with reduced risk of breast cancer. Triterpenoids are one of the largest groups of phytochemicals. 2α-Hydroxyursolic acid, as a member of triterpenoids family, has been isolated from apple peels previously. It was reported that 2α-hydroxyursolic acid displayed anti-cancer effect against several human cancer cell lines. However, the underlying mechanisms of actions of 2α-hydroxyursolic acid in the prevention of cancer were not fully understood. The goal of this study is to investigate the mechanisms of 2α-hydroxyursolic acid in inhibiting cell proliferation, inducing of cell apoptosis and suppressing cell metastasis in human MDA-MB-231 cells. 2α-Hydroxyursolic acid significantly inhibited MDA-MB-231 cells proliferation in a dose-dependent manner, and no cytotoxicity was observed at the concentrations below 30 μM. 2α-Hydroxyursolic acid significantly down-regulated expression of TRAF2, PCNA, Cyclin D1, and CDK4 and up-regulated the expression of p-ASK1, p-p38, p-p53, and p-21. 2α-Hydroxyursolic acid induced apoptosis in MDA-MB-231 cells by

significantly increasing the Bax/ Bcl-2 ratio and inducing the cleaved caspase-3. For the effect on cell metastasis, 2α-hydroxyursolic acid significantly inhibited hepatocyte growth factor (HGF)-induced and epidermal growth factor (EGF)-induced MDA-MB-231 cell migration, invasion and colony formation. Western blot analysis indicated that 2α-hydroxyursolic acid significantly inhibited HGF-induced phosphorylation of Met and Akt, nuclear protein levels of NF-κB and VEGF expression. Furthermore, the HGF-induced activity of MMP-2, critical enzymes for cancer cell migration and invasion, was dramatically inhibited in a dose-dependent manner. 2α-Hydroxyursolic acid significantly inhibited EGF-induced phosphorylation of EGFR and Akt, nuclear protein levels of NF-κB, c-Jun and c-Fos, as well as VEGF expression. Furthermore, the EGF-induced gelatinolytic activities of MMP-2 and MMP-9 were dramatically inhibited in a dose-dependent manner. These results suggested that 2α-hydroxyursolic acid exhibited anti-cancer activity through the inhibition of cell proliferation, induction of apoptosis and the inhibition of cell metastasis.

BIOGRAPHICAL SKETCH

Xue Jiang is a Ph.D. student in the Department of Food Science at Cornell University. Before joining the Department of Food Science at Cornell University, Xue received a bachelor degree of Food Science and Technology in the Department of Beijing Forestry University. In 2011, Xue Jiang was admitted to a graduate program in food science at Cornell University. During the past 5 years, she has been taking coursework and conducting research in the area of bioactive food components in cancer chemoprevention under the guidance of Professor Rui Hai Liu. The major contribution of her research works is to reveal inhibitory effect of 2α-hydroxyursolic acid, a natural compound isolated from apple peels, against breast cancer. In the final year of her Ph.D. study, she received the Goya Foods Prize which recognizes her achievements/contribution in the area of fruits and vegetables. Her goal is to apply what she has learnt in this area to lead society aware a healthier life.

Beyond of academics, Xue actively participates in various University activities. She was selected to join the Cornell Student Multidisciplinary Applied Research Team (SMART) Program which provided her the opportunity to assist in the market plan design for a supplement company in Thailand. In addition, she took part a start-up competition of the Johnson Business School to promote ‘ready-to-go slow-cooker meals’. Xue enjoys outside activities including ice skating, snowboarding and badminton.

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LIST OF ABBREVIATION

5-FU, 5-fluorouracil;

AMR-Me, Methyl 25-Hydroxy-3-oxoolean-12-en-28-oate;

AP-1, activator protein-1;

ASK, apoptosis signal regulating kinase;

Bax, Bcl-2 associated X protein;

Bcl-2, B cell lymphoma-2;

BRCA1, breast cancer–associated gene 1;

Brk, Breast tumor kinase;

CAM, chorioallantoic membrane;

Caspase, cysteine aspartic acid specific protease;

CDDO, 9 (11)-dien-28-oic acid;

CDDO-EA, CDDO-ethyl amide;

CDDO-Im, CDDO-imidazolide;

CDDO-Me, CDDO-methyl ester;

CDK, Cyclin-dependent kinase;

C_{max}, maximum plasma concentration;

ECM, extracellular matrix;

EGF, epidermal growth factor;

EGFR, epidermal growth factor receptor;

EMT, epithelial-mesenchymal transition;

ER, estrogen receptor;

ERK1/2, extracellular-signal-regulated kinase1/2;

FBS, fetal bovine serum;

FoxM1, Forkhead box protein M1;

Grb2, growth factor receptor-bound protein 2;

HBSS, Hank's balanced salt solution;

HER2, human epidermal growth factor receptor 2;

HGF, hepatocyte growth factor;

HIF-1α, hypoxia-inducible factor-1α;

HIMOXOL, Methyl 3-hydroxyimino-11-oxoolean-12-en-28-oate;

ICAM-1, intercellular adhesion molecule-1;

IGF, insulin-like growth factor;

IGFBP1, insulin-like growth factor binding protein 1;

IKK, IκB kinase;

IL-1β, interleukin-1 β;

IL-8, interleukin-8;

JAK1, Janus-activated kinase 1;

JAK2, Janus-activated kinase 2;

JNK1/2, c-Jun N-terminal kinase1/2;

MAPK, mitogen activated protein kinase;

MDSC, myeloid-derived suppressor cells;

MMPs, matrix metalloproteases;

NF-κB, nuclear factor kappa B;

NG, O(2)-(2,4-dinitro-5-{[2-(12-en-28- β -D- galactopyranosyl-oleanolate-3-yl) -oxy-2-oxoethyl]amino}phenyl)1-(N-hydroxyethylmethylamino)diazen-1-ium-1,2- diolate;

PARP, poly ADP ribose polymerase;

PCNA, proliferating cell nuclear antigen;

PI3K, phosphatidylinositol-3-kinase;

PKC- ξ, protein kinase C- ξ;

PR, progesterone receptor;

PyMT, polyoma virus middle T oncoprotein;

Rac1, Ras-related C3 botulinum toxin substrate 1;

RhoA, Rho-like GTPases;

SMEDDS, self-microemulsifying drug delivery system;

$t_{1/2}$, the elimination half-life;

TBST, Tris-base buffer solution containing 0.1% Tween-20;

t_{max}, The time to peak plasma concentration;

TRAF, tumor necrosis factor receptor-associated factor;

TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling;

uPA, urokinase-type plasminogen activator;

VEGF, vascular endothelial growth factor;

α-MEM, α-Minimum Essential Medium.

CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Breast cancer

Cancer is a major health problem worldwide. A total of 1,685,210 new cancer cases and 595,690 cancer deaths are predicted to occur in the United States in 2016 [1]. Even though death rates nationwide have a slight decrease during the recent 5 years in both men and women, cancer currently is expected to overtake heart disease as the leading cause of death in the next few years [1]. Indeed, it is predicted that by 2020 approximately 15,000,000 new cancer cases will be diagnosed worldwide and 12,000,000 people will die from cancer [2]. The most common diagnosed cancers will be prostate, lung and bronchus, and colorectal cancers in men and breast, lung and bronchus, and colorectum cancers in women [1]. As the most common cancer diagnosed among women in the United States, breast cancer alone accounts for nearly 30% cancers in women. Breast cancer is also the leading cause of cancer death in women aged 20 to 59 years [3]. A total of 231,000 new cases of invasive breast cancer and 40,000 breast cancer deaths were expected to occur among US women in 2015 [4]. Traditional therapies include surgery, radiation and chemotherapy have many side effects that affect patients' quality of life post-treatments. Therefore, it is necessary to develop novel preventive and therapeutic approaches for breast cancer [5]. Chemoprevention, considered as the prevention, alleviation or reversion of

carcinogenesis by the administration of one or more naturally occurring and/or synthetic compounds, is one of the promising approaches [6-9]. The most common chemoprevention agents for breast cancer include human epidermal growth factor receptor 2 (HER2)-targeting antibody (trastuzumab and lapatinib), estrogen receptor (ER) modulator (tamoxifen and raloxifene) and vascular endothelial growth factor (VEGF)-targeting antibody (bevacizumab) [6, 10-13]. However, alleviation of side effects induced by these chemoprevention agents is limited. Triple-negative breast cancer, which accounts for approximately 15–20 % of all breast cancers, cannot be effectively targeted by these chemoprevention agents. Triple-negative breast cancer is defined as tumors that are ER-negative, PR-negative, and HER2-negative [14, 15].

1.1.2 Phytochemicals

It is estimated that 5000 individual phytochemicals have been identified from fruits, vegetables, grains. Many of them are being developed as natural chemoprevention agents for chronic disease including breast cancer [16, 17]. Phytochemicals are secondary metabolites secreted in plants in response to a variety of environmental stressors, such as ultraviolet (UV) light, invading microorganisms and insects, and drought [18]. Epidemiological studies showed that increased consumption of rich phytochemicals from food, such as, grains, cereals, vegetables, and fruits, can provide a significant inverse association with breast cancer [19-25].

1.1.3 Triterpenoids

Triterpenoids, synthesized in many plants [26], represent the largest group of phytochemicals [9]. More than 20,000 triterpenoids are known to occur in nature [27]. Triterpenoids are ubiquitous compounds in our diet, especially plant-based food [28]. Triterpenoids are considered relatively non-toxic to humans and display a wide range of important medicinal activities, including anti-inflammatory [29, 30], anti-tumor [8, 31, 32], anti-bacterial [33], anti-HIV [34-36] and antidiabetic [37, 38] activities. Triterpenoids can be classified into diverse groups including squalene group, lanostane group, dammarane group, hopane group, lupane group, oleanane group and ursane group [39].

Ursolic acid, 2α-hydroxyursolic acid, oleanolic acid, and their derivatives belong to the triterpenoids family and have been human disease. In this review, the natural sources, bioavailability, and distribution of ursolic acid, 2α-hydroxyursolic acid, oleanolic acid are introduced. Both *in vitro* and *in vivo* effects of the three mentioned triterpenoids as well as their derivatives in cancers, especially breast cancer, are discussed based on the research conducted recently.

1.2 Major sources

1.2.1 Ursolic acid

Ursolic acid ($C_{30}H_{48}O_4$) is pentacyclic triterpene acids, may occur as free acid or as aglycone of saponins [40]. Ursolic acid not only exists in variety of medicinal herbs, but also has been found in fruits and vegetables. Those are fruits of *Crataegus*

pinnatifida var. psilosa [41], basil (*Ocimum basilicum*), brown mustard (*Brassica juncea*), daylily (*Hemerocallis f ulva* L.), balsam pear (*Momordica charantia*), mahogany (*Toona sinensis*), calamondin (*Citrus microcarpa* Bonge), guava (*Psidium guajava*) [42], lingonberry (*Vaccinium vitis-idaea* L.) [43], Cranberry fruit (*V. macrocarpon*, var. Early Black), Cranberry fruit (*V. macrocarpon*, var. Howes), Cranberry fruit (*V. macrocarpon*, mixed var.), Lowbush blueberry (*V. angustifolium*), Northern cranberry (*V. oxycoccus*), Partridge berry (*V. vitis-idaea*) [44], and cowberry (*Vaccinium vitis-idaea* L.) [45].

1.2.2 2α-Hydroxyursolic acid

2α-Hydroxyursolic acid ($C_{30}H_{48}O_3$), also named corosolic acid, was first isolated from fruits of *Crataegus pinnatifida var. psilosa* [41]. Other fruits and vegetables sources including apple peel [46], *Hippophae rhamnoides* [47], mulberry (*Mours alba* L.), basil (*Ocimum basilicum*), mahogany (*Toona sinensis*) and daylily (*Hemerocallis fulva* L.) [42]. 2α-Hydroxyursolic acid has also been discovered in various traditional Chinese medicinal herbs. These include *Lagerstroemia speciose* [48], *Eriobotrta japonica* [49], *Tiarella polyphyll* [50], and *Potentilla discolor Bunge* [38].

1.2.3 Oleanolic acid

Oleanolic acid ($C_{30}H_{48}O_3$), an oleanane-type pentacyclic triterpenoid, is an isomer of ursolic acid. Oleanolic acid widely distributed in dietary plants, medicinal herbs and other plants, has been isolated from more than 1600 plant species [51, 52], including

olive cultivars (*Olea europaea*, L.) [53], Grape (*Vitis vinifera*) [54], cowberry (*Vaccinium vitis-idaea* L.) [45], basil (*Ocimum basilicum*) daylily (*Hemerocallis f ulva* L.), mahogany (*Toona sinensis*), calamondin (*Citrus microcarpa* Bonge) [42]. Due to the low bioavailability, oleanolic acid was used as the starter for synthesizing 2-Cyano-3, 12-dioxooleana-1, 9 (11)-dien-28-oic acid (CDDO), CDDO-methyl ester (CDDO-Me), CDDO-imidazolide (CDDO-Im), CDDO-ethyl amide (CDDO-EA), which are the most representative synthetic oleanane triterpenoids that possess several promising pharmacological effects [27, 55].

1.3 Bioavailability and distribution

1.3.1 Ursolic acid

In an animal study, the bioavailability of ursolic acid was determined using LC-MS method by Liao et al. [56]. Lu-Ying (*Sambucus chinensis* L.) extract containing 80.32 mg/kg ursolic acid was administered to rats by oral gavage. The blood samples were collected from the abdominal vein before 0 h and at 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 12.0 h after administration. The results showed that ursolic acid was absorbed rapidly, with the peak concentration occurring 1.0 h after oral administration of Lu-Ying extract. The elimination half-life ($t_{1/2}$) was 4.3 h and the maximum plasma concentration (C_{max}) was 294.8 ng/ml, which was extremely low although the high oral administration dose of Lu-Ying contained ursolic acid. This implied that ursolic acid may have high binding activity in organs and low blood distribution.

Due to the poor solubility of ursolic acid, efforts have been devoted to improve

its bioavailability and increase therapeutic efficiency. Zhou et al. [57] prepared and evaluated ursolic acid phospholipid nano-powders, which had a good targeting to the liver compared to ursolic acid itself after intravenous administration to Male Kunming mice. Two other studies characterize the pharmacokinetics of ursolic acid in human after intravenous infusion of ursolic acid liposomes [58]. In particular, Xia et al.[58] developed a rapid, sensitive and specific UPLC/MS/MS method to determine the total ursolic acid in human plasma. Eight healthy volunteers were treated with intravenous infusion of ursolic acid nano-liposomes at the dose of 98 mg/m^2. C_{max} was 3404.6 ± 748.8 ng/ml, $t_{1/2}$ was 3.9 ± 2.1 h and AUC 0→t was 9644.1 ± 1193.2 ng•h/ml. In the study conducted by Wang et al. [59], following 37, 74, and 98 mg/m^2 infusion in 24 healthy adult volunteers, C_{max} was, 1835 ± 438, 2865 ± 868, and 3457 ± 856 ng/mL, respectively. The time to peak plasma concentration (t_{max}) was, respectively, 4.03 ± 0.04, 4.02 ± 0.04, and 4.0 ± 0.00 h. All of the studies above demonstrated the potential of modern technology to overcome the poor solubility of ursolic acid.

The distribution of ursolic acid in tissues after absorption has been evaluated by previous studies. Chen et al. [60] measured the tissue distribution of ursolic acid in the rats at 1.0 h after 10 mg/kg dose by oral administration. The observed distribution consequence in ursolic acid concentration was as follow: lung>spleen>liver>cerebrum>heart>kidney. The result implied that the distribution of ursolic may depend on the blood flow and perfusion rate of the organ since ursolic acid is mainly distributed in blood abundant tissues such as lung, spleen and liver. However, the concentration in kidney is the lowest compared to other organs. It indicates that kidney might not be the primary excretion organ of prototype ursolic

acid [60]. In another study, mice were fed a normal diet plus 0.5% ursolic acid, prepared by mixing 0.5 g of target compound with 99.5 g of powder diet. Instead of measuring after a short-term, the concentrations were measured in different tissues after a few weeks. The results showed as follow: liver > colon > kidney > heart > bladder > brain. The level of ursolic acid in each organ increased as the feeding period was increased from 4 weeks to 8 weeks. The liver has the highest content of ursolic acid which implies that liver is the major organ for ursolic acid storage and/or metabolism. The result indicated that the intact form of ursolic acid was available in tissues after oral intake, which ensures its deposition and ability to exert local or systemic protective effects and actions. This also indicates the importance of measuring the health benefits of intact ursolic acid *in vitro* [42].

1.3.4 2α-Hydroxyursolic acid

2α-Hydroxyursolic acid might be poorly absorbed in the small intestine and rapid metabolized, leading to low oral bioavailability. It could be detected in circulation and/or organs after ingestion [42]. Liu et al. [37] first reported the oral bioavailability of 2α-hydroxyursolic acid in rats after 20 mg/kg dosage. 5, 10, 20, 30, 45, 60, 90, 120, 150, 180 min after the oral administration in the form of pure compound, blood samples were collected. The results showed a single-peak profile at 9.2 min with peak plasma concentration of 0.30 ± 0.19 μg/ml, the oral bioavailability is 0.93%. Li et al. [38] via HPLC-MS methods also determined the 2α-hydroxyursolic acid concentration in plasma samples of normal rats. In this study, rats were orally administered with the

1.33 g/kg extract of *P. discolorat* instead of pure compound. A double-peak profile around 0.5 and 2 h, with peak concentrations around 0.25 and 0.27 μg/mL. In the same study conducted by Yin et al. [42], 0.5% of 2α-hydroxyursolic acid was fed to mouse. 8 weeks later, there was no significant difference of ursolic acid in organs except there is higher concentration of 2α-hydroxyursolic acid in liver tissue.

1.3.5 Oleanolic acid

A sensitive HPLC–ESI–MS–MS method was established for the pharmacokinetics of oleanolic acid. Eighteen healthy male Chinese volunteers were administrated with 40 mg capsules of oleanolic acid. The maximum plasma concentration was 12.12 ± 6.84 ng/ml, which is extremely low partially due to the low consuming dose [61]. The pharmacokinetics of oleanolic acid was also evaluated in another study conducted by Jeong et al. [62]. In this study, oleanolic acid dissolved in a mixture of *N, N*-dimethylacetamide:PEG400:water (2:4:1, v/v) was administered to rats at doses of 10, 25 and 50 mg/3 ml/kg by oral gavages. The maximum plasma concentration was 74.0 ± 57.2 ng/ml for 25 mg/kg dose and 132.0 ± 122.0 ng/ml based on 50 mg/kg dose. The absolute oral bioavailability was 0.7% for oral doses of 25 and 50 mg/kg. The very low oral bioavailability of oleanolic acid could be due to a poor absorption and extensive metabolic clearance [62]. In order to enhance the oral bioavailability of oleanolic acid, a self-microemulsifying drug delivery system (SMEDDS) was adopted by Yang et al. [63]. SMEDDS successfully increased oral bioavailability of oleanolic acid by 5.07-fold compared with the marketed product in tablet form. SMEDDS consists of oil, surfactant, cosurfactant and drug. In this study, the recommended

formulation is 50% ethyl oleate as the oil, 35% Cremophor EL as the surfactant, and 15% alcohol as the cosurfactant [63]. In another study conducted by Jiang et al. [64], a solidified phospholipidcomplex (OPCH) composed of oleanolic acid-phospholipid complex (OPC) to increase the liposolubility and hydroxyapatite (HA) was prepared to improve the flowability. When co-administrated of Ketoconazole, an inhibitor of oleanolic acid metabolism, OPCH significantly improved the bioavailability of oleanolic acid [64].

The organ distribution and content of oleanolic acid after oral administration are similar with ursolic acid according to the study of Yin et al. as we discussed above [42]. Liver is also the predominant organ for storage and/or metabolism of oleanolic acid.

From the studies I discussed above, bioavailability of the traditional triterpenoids were really low, which may due to their low solubility in aqueous medium. Therefore, introducing modern technology, seeking for new natural triterpenoids and synthesizing novel triterpenoids may help mitigate the effects of breast cancer.

1.4 Triterpenoids and breast cancer

Substantial studies have been done to elaborate the therapeutic and preventive effects of triterpenoids in breast cancer [8, 9]. In the following sections, the anti-cancer effect of three representative triterpenoids: ursolic acid, 2α-hydroxyursolic acid, oleanolic acid, and their derivatives will be discussed based on research mainly published in recent five years.

1.4.1 In vitro study

In vitro studies that demonstrate the effects of ursolic acid, 2α-hydroxyursolic, oleanolic acid, and their derivatives on inhibiting proliferation, cell cycle, metastasis and inducing apoptosis of different cancer cell lines as well as the underlying mechanisms are summarized.

1.4.1.1 Effects on the inhibition of proliferation and cell cycle regulation

It has been well accepted that cancer is a disease with a hyper-proliferative disorder. Cancer cells own the ability to reach the barrier which limits the proliferative potential, and accumulate in cluster which leads to tumor formation [65, 66]. Cell cycle could be divided into five phases: G1 (first gap), S (during which DNA replication takes place), G2 (during which cell growth continues and proteins are synthesized), M (mitosis, namely nuclear division) and G0 (during which cells are resting, non-proliferative). G1 is an essential phase, during which cells make critical decisions about growth versus quiescence [66]. Major control switches of the cell cycle are the Cyclins and the Cyclin-dependent kinases. Proliferating cell nuclear antigen (PCNA) is often used as a proliferation marker. Cyclin D1, a component subunit of Cyclin-dependent kinases 4 (CDK4) and CDK6, is a rate-limiting factor in progression of cells through the first gap (G1) phase of the cell cycle [67]. p21, as CDK inhibitor, involved in the negative regulation of cell cycle progression from G1 to S phase [68].

There are numerous *in vitro* studies that demonstrate the inhibitory effects of ursolic acid, 2α-hydroxyursolic and oleanolic acid against proliferation in a large variety of cancer cell lines.

Lin et al. [69] examined whether ursolic acid could inhibit the proliferation of HT-29 colorectal cancer cells, and the mechanism it employs. The results showed that the proliferation of HT-29 cells were suppressed in a dose-dependent manner after 24 h treatment of ursolic acid and the percentage of HT-29 cells in S-phase was decreased. Both mRNA and protein levels of cell cycle regulator: Cyclin D1 and CDK4 were significant decreased by ursolic acid treatment, while mRNA and protein levels of p21 were significantly increased after UA treatment [69]. Using SNG-2 and HEC108 endometrial adenocarcinoma cancer cells, ursolic acid also inhibit the expression of cyclin D1 through modulating MAPK signaling pathways [70]. The effect of modulation of cell cycle was also observed for oleanolic acid. Flow cytometry assay showed that oleanolic acid induced G0/G1 arrest and decreased percentage of cells in the S phase in PC-3, DU145, and LNCaP prostate cancer cells. The cell cycle arrest was accompanied by the decrease of the expression levels of CDK4, and cyclin D1, as well as PI3K and p-Akt, indicating the involvement of PI3K/Akt pathway in oleanolic acid modulated cell cycle distribution [71]. Similarly, HepG2 human liver cancer cells were found to accumulate in G0/G1 phase after 24 h treatment of oleanolic acid, while the number of cells in the S and G2/M phases was markedly reduced. Furthermore, a dose‒ and time‑dependent inhibition of oleanolic acid were observed using an MTT assay [72]. However, another study also conducted in HepG2 human

liver cancer cells showed a G2/M cell cycle arrest induced by oleanolic acid with a decrease of mRNA expression of two important G2/M phase transition checkpoints: cdc2 and cyclin B1, as well as the increase the expression of p21, p53 and ERK phosphorylation. PFT-α, an inhibitor of p53, significantly abolished oleanolic acid-induced expression of p21 and cell cycle arrest. Similarly, in the presence of U0126, an inhibitor of MEK/ERK signaling, the upregulation of p53 expression by oleanolic acid was significantly inhibited in HepG2 cells, indicating a key role of ERK in the initiation of cascades responsible for oleanolic acid-modulated cell cycle arrest [73].

Cell proliferation was suppressed through multiple signaling pathways which is cell-type dependent. Ursolic acid inhibited the proliferation of HT-29 colon cancer cells by suppressing the phosphorylation of EGFR in a dose- and time-dependent manner, with downregulation the expression of MAP Kinases including ERK1/2, p38 MAPK, and JNK in HT-29 cells [74]. In a study on CAOV3 human ovarian cancer cells, ursolic acid inhibited cell proliferation via decreasing the expression of p-ERK1/2 and increasing MKP-1, a negative regulator of ERK [75]. p38 MAPK was detected to play an important role in ursolic acid-induced anti-proliferative activity and activation of insulin-like growth factor (IGF) binding protein 1 (IGFBP1) and human forkhead box class O 3a (FOXO3a) transcription factor in Bel-7402 and HepG2 hepatocellular carcinoma cells [76]. Oleanolic acid was reported to inhibit cell proliferation of T24 human bladder cancer cells through targeting on Akt/mTOR/S6K and ERK1/2 signaling pathways [77].

Transcriptional factors such as signal transducers and activators of transcription 3 (STAT3) and nuclear factor kappa-B (NF-κB) are reported to be

suppressed by these triterpenoids resulting in inhibition of proliferation in a variety of cancer cell lines. Pathak et al. [78] indicated that DNA binding activity of STAT3, constitutive and interleukin-6 (IL-6)-inducible STAT3 phosphorylation were inhibited by ursolic acid in U266 human multiple myeloma cells resulting in inhibition of cell proliferation. Ursolic acid also caused G0/G1 arrest and down-regulated the expression of STAT3-regulated gene products, including cyclin D1, surviving. The upstream kinases c-Src, Janus-activated kinase 1 (JAK1), Janus-activated kinase 2 (JAK2), and ERK ½, but not Akt were reported to involve in the STAT3 suppression by ursolic acid [78]. Using androgen-independent (DU145) and androgen-dependent (LNCaP) prostate cancer cell lines, Shanmugam et al. [79] investigated that if ursolic acid inhibited cell proliferation via NF-κB and STAT3 signaling pathways. MTT assay indicated that treatment with ursolic acid inhibited cell proliferation in a dose- and time-dependent manner in both cell lines, but a stronger inhibitory effect was observed in LNCaP cells. Ursolic acid inhibited TNF-α-induced IκBα phosphorylation, p65 phosphorylation, activation of IKK, NF-κB DNA binding activity and IL-6-inducible STAT3 and JAK2 phosphorylation in LNCaP cells. While in DU145 cells, ursolic acid inhibited constitutive IκBα phosphorylation, p65 phosphorylation, NF-κB DNA binding activity, STAT3 phosphorylation and JAK2 kinase [79]. More recently, ursolic acid was reported to inhibit proliferation in human pancreatic cancer AsPC-1, MIA PaCa-2, and Panc-28 cell lines. After 8 h treatment in Panc-28 cell, ursolic acid inhibited constitutive NF-κB activation and its regulated gene products including the expression of cyclin D1 in a dose-dependent manner [80]. Transcriptional factors were also reported to involve in 2α-hydroxyursolic acid induced anti-proliferative activity.

The studies conducted by Fujiwara et al. [81, 82] revealed that 2α-hydroxyursolic acid inhibited U373 glioblastoma cell proliferation by suppressing the constitutive activation of STAT3 and NF-κB at a concentration of 20 μM or higher [81], and inhibited SKOV3, RMG-1 and ES-2 epithelial ovarian cancer cells proliferation through suppressing STAT3 activation at a concentration of 30 μM or higher [82].

The studies on MCF-7 human breast cancer cell line revealed that ursolic acid induces cell cycle arrest and inhibit cell proliferation. Wang et al. [83] assessed the effect of ursolic acid on proliferative inhibition and cell cycle dysregulation in MCF-7 human breast cancer cells. MTT test showed that ursolic acid inhibited the proliferation of MCF-7 cells in a dose and time-dependent manner. The mechanism of anti-cancer effect of ursolic acid could be attributed to the inhibitive effect of both gene expression and protein expression of Forkhead box protein M1 (FoxM1), an essential transcription factor for cell cycle progression and CyclinD1/CDK4. Ursolic acid significantly increased the cell number in G1 phase in the concentration range of 10-17.5 μM through the induction of p21 and p53 [84]. In another study, the anti-proliferative and associated mechanisms of a few pentacyclic triterpenes, including ursolic acid, 18α-glycyrrhetinic acid, carbenoxolone and dimethyl melaleucate have been investigated in breast cancer cells *in vitro* [85]. The results showed that these pentacyclic triterpenes are able to inhibit EGF-induced cell proliferation in MCF-7, as well as MDA-MB-231 breast cancer cells lines. Epidermal growth factor receptor (EGFR), a receptor expressed in about 30% of breast cancers, has always been considered as a potential target for breast cancer, especially triple negative breast cancer. All the pentacyclic triterpenes used in the study suppressed the

phosphorylation of EGFR through ATP binding pocket of EGFR kinase domain as well as the downstream effectors STAT3 and Cyclin D1 in both breast cancer cell lines [86].

2α-Hydroxyursolic acid showed anti-proliferative effect against human breast cancer cells. Yoon et al. [87] treated MCF-7 human breast cancer cells with different doses of 2α-hydroxyursolic acid for 24 h. The result showed that 2α-hydroxyursolic acid significantly inhibited MCF-7 cell proliferation at doses of 20 μM ($p<0.05$). In addition, 2α-hydroxyursolic acid suppressed the activation of NF-κB, which is in charge of the expression of genes related to many physiological processes including cell proliferation [87]. We also investigated the mechanisms of action of 2α-hydroxyursolic acid in inhibiting cell proliferation and inducing apoptosis in a triple-negative breast cancer cell line, MDA-MB-231 human breast cancer cells. In this study, 2α-hydroxyursolic acid up-regulated the expression of p21, a CDK inhibitor and down-regulated the protein expression of CDK4, Cyclin D1 and PCNA. Upstream proteins as TNF receptor associated factor 2 (TRAF2), phosphorylated apoptosis signal-regulating kinase 1 (p-ASK1), phosphorylated p38 and p53 are also being regulated by 2α-hydroxyursolic acid. p-38 specific inhibitor, SB203580 had also been used to conclude that 2α-hydroxyursolic acid targeted the p38 MAPK pathway to inhibit cell proliferation and induce apoptosis in MDA-MB-231 human breast cancer cells [88]. Except for 2α-hydroxyursolic acid, ursolic acid and 11 other triterpenoids were isolated from apple peels, they showed strong anti-proliferative activities against human HepG2 liver cancer cells, MCF-7 breast cancer cells, and Caco-2 colon cancer cells which suggested that apple peels proves to be pharmaceutically beneficial [46].

Oleanolic acid has been reported to inhibit proliferation in MCF-10A human mammary epithelial cells and MDA-MB-231 human breast cancer cells, but not in MCF-7 human breast cancer cells [89].

Ursolic acid and oleanolic acid are also the two most effective compounds from *Oldenlandia diffusa*, a well-known Chinese medicinal plant. Both ursolic and oleanolic acids inhibited cell growth correlated with increasing p53 and $p21^{WAF1/Cip1}$ protein expression in MCF-7 breast cancer cells. In addition, presence of ursolic and oleanolic acids strongly reduced cell viability in tamoxifen-resistant cells, suggesting a potential role for these compounds in breast cancers developing hormone resistance [90]. While, according to an earlier study conducted by Amico et al. [91], ursolic acid showed higher anti-proliferative activity toward MCF-7 cells than both oleanolic acid and 2α-hydroxyursolic acid. Compounds were purified from almond hulls (*P. dulcis*, Pizzutella variety) and were tested with MTT assay [91]. This study is partially consistent with another study, in which, ursolic acid was more effective in inhibiting anti-proliferation than oleanolic acid in both MCF-7 and MDA-MB-231 breast cancer cell lines through MTT assay [92].

Results from *in vitro* studies suggested that the triterpenoids and their derivatives significantly inhibit the growth of cancer cells including breast cancer cell lines. However, it is found that the performance among different compounds is inconsistent. For instance, one study demonstrated that ursolic acid has stronger inhibitory effect than oleanolic acid and 2α-hydroxyursolic acid in MCF-7 cells, while another study showed that 2α-hydroxyursolic acid has superior anti-proliferative effect over ursolic acid. One possible reason could be the different assays they adopted.

1.4.1.2 Effects on the induction of apoptosis

Apoptosis, or programmed cell death, is a check and balance process that the cells use to maintain homeostasis [93]. Apoptosis could be triggered through the activation of either intrinsic (mitochondrial) pathway or extrinsic (receptor-activated) pathway [94]. The intrinsic pathway is initiated by signals within the cell. In the intrinsic pathway, cytochrome c is released from mitochondria to cytosol as a result of depolarization of the outer mitochondrial membrane. The release resulted in formation of apoptosome, which converts a potential cytoplasmic protease procaspase 9 to its active form, cleaved caspase 9 and thereby cleaves procaspase 3 [66, 95, 96]. The sequential protease cleavage resulted apoptosis eventually. Activation of intrinsic pathway is regulated by Bcl-2 family members. Bcl-2, an anti-apoptotic protein, negatively regulates the leakage of cytochrome c by keeping the mitochondrial channel closed [97]. Bax, a pro-apoptotic member, favors the leakage of cytochrome c thus accelerating programed cell death [98]. The extrinsic pathway is induced by the signals outside the cell. In the extrinsic pathway, ligands are fixed to death receptors which present on the cell surface. Death receptor belongs to the tumor necrosis factor (TNF) receptor family, such as Fas or TNF receptor-1 (TNFR1). This attachment bypasses the mitochondrial step and stimulates the formation of the death inducing-signaling complex, which releases caspase-8, and thereby activates downstream caspases, such as caspase-3, -6 or -7, without any involvement of the BCL-2 family [96, 99].

Ursolic acid induced apoptosis mainly through intrinsic pathway. The study on T24 human bladder cancer cells revealed that pretreatment with ursolic acid activated AMPK pathway via increasing cellular ceramide level, which thereby mediated JNK activation, mTORC1 inhibition, survivin downregulation, caspase-3 and its downstream poly ADP ribose polymerase (PARP) cleavage, thus resulted in apoptosis [100]. Park et al. [101] reported that ursolic acid downregulated the expression of Bcl-X_L , Bcl-2 and Mcl-1, upregulated the expression of Bax, cleaved caspase-9, caspase-3 and PARP through Wnt5/GSK3β/β-catenin signaling in PC-3 prostate cancer cells [101]. Ursolic acid induced apoptosis through PI3K/Akt pathway in K562 human leukemia cells, characterized by an increase in cytochrome c in the cytosol fraction, with significant increase of cleaved caspase-9 and caspase-3 expression [102]. Shan et al. [103] and Wang et al. [104] demonstrated that in SW480 colon cancer cells, ursolic acid induced apoptosis through intrinsic pathway, suggesting ursolic acid as a promising candidate for the prevention and treatment of human colon cancer. Similar effects of ursolic acid have been observed in other cell lines include HT-29 colorectal cancer cells [69], LNCaP and PC-3 prostate cancer cells [105], HepG2 hepatocellular carcinoma cells [106], gastric cancer SGC-7901 cells [107], Me4405 human melanoma cells [108]. While, ursolic acid could also induce extrinsic apoptotic pathway in several cell lines. According to a study conducted by Wang et al. [109], ursolic acid induced apoptosis through both intrinsic and extrinsic apoptotic pathways in BGC-803 gastric cancer cells. It was proved by the data that expression of pro caspase-3, -8, and -9 proteins declined after UA treatment, indicating that activated caspase-3, -8, -9 proteins were induced by UA treatment. Expression of Bcl-2 protein

was also downregulated by UA treatment [109]. Ursolic acid induced extrinsic apoptotic pathway has also been observed in MDA-MB-231 human breast cancer cells [110], which will be discussed later.

Ursolic acid is known to sensitize tumor cells to a number of chemotherapeutic agents. In a study conducted by Shin et al. [111], ursolic acid sensitized LNCaP human prostate cancer cells, to undergo a necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Specifically, co-treatment with 20 μM ursolic acid and 50 ng/ml TRAIL caused cell shrinkage, apoptotic bodies, and detachment from plates, as well as the modulation of the expression of key apoptosis marker proteins including caspase-3, caspase-8, caspase-9, PARP, XIAP and Bid. Another study carried out by Xavier et al. [112] revealed that a significant enhancement of apoptosis was observed when ursolic acid combined with with 5-fluorouracil (5-FU) in a apoptosis-resistant HCT15 colorectal carcinomas cells. Furthermore, ursolic acid also modulated autophagy by enhancing the accumulation of LC3 and p62 levels through JNK pathway in HCT15 cells. Combined ursolic acid with melatonin has also been reported to trigger the release of cytochrome c and induce the cleavage of caspase and PARP proteins in SW480 human colon cancer cells [113].

Also, 2α-hydroxyursolic acid exhibits strong pro-apoptotic activity by activating both intrinsic and extrinsic pathways. In HCT116 human colon cancer cells, 2α-hydroxyursolic acid induced cell apoptosis, characterized by the morphological changes and chromatin condensation [114]. The expression of molecular proteins linked with intrinsic pathway (Bax, bcl2, caspase-9 and caspase-3) and extrinsic pathway (Fas/Fas ligand, caspase-8 and PARP) were found to be altered by 2α-

hydroxyursolic acid. Similar mechanisms were proposed on 2α-hydroxyursolic acid induced cell apoptosis in HL-60 human promyelocytic leukemia cells [115], MG-63 human osteosarcoma cells [116] and HeLa human cervix adenocarcinoma cells [117]. ROS plays an important role in the regulation of apoptosis [118]. Using A549 lung adenocarcinoma cells, Nho et al. [119] reported that 2α-hydroxyursolic acid induced apoptosis via the generation of ROS along with the alteration of mitochondrial transmembrane potential and the expression of apoptosis-related proteins, induction of cytochrome c release from mitochondria and caspases [119].

While apoptosis induced by oleanolic acid seems to be only mitochondrial-dependent. Oleanolic acid induced intrinsic apoptosis pathway in GBC-SD and NOZ in gallbladder cancer cells [120], HepG2 human hepatocellular carcinoma cells [72, 73] and Panc-28 human pancreatic cancer cells [121]. Some tumor cell lines are resistant to the pro-apoptotic effect of oleanolic acid [122], oleanolic derivatives exhibited stronger effect including 2-(pyrrolidine-1-yl) methyl-3-oxoolean-12-en-28-oic acid (SZC014) in SCG7901 gastric cancer cells [123], CDDO-Me in MiaPaCa-2 and Panc-1 pancreatic cancer cells[124], Methyl 3-hydroxyimino-11-oxoolean-12-en-28-oate (HIMOXOL) in MDA-MB-231 breast cancer cells [125], O(2)-(2,4-dinitro-5-{[2-(12-en-28-β-D- galactopyranosyl-oleanolate-3-yl) -oxy-2-oxoethyl]amino}phenyl)1-(N-hydroxyethylmethylamino)diazen-1-ium-1,2- diolate (NG) in HepG2 human hepatocellular carcinoma cells [126].

The upstream protein regulators involved in these triterpenoinds inhibited cell apoptosis are complex and probably cell-type-dependent, including AMPK pathway [100], Akt/NF-κB [127] and ASK1–JNK in T24 human bladder cancer cells [128],

PI3K/Akt/mTOR pathway [105] and Akt/NF-κB/mTOR pathway [129] in LNCaP and PC-3 human prostate cancer cells, AMPK in HepG2 hepatocellular carcinoma cells [106] and SNU-601 human gastric cancer cells [130], JNK and AKT pathway in MKN28 gastric cancer cells [131], ERK/Nrf2/ROS pathway in A549 lung cancer and PANC-1 pancreatic carcinoma cells [132].

Ursolic acid has been reported to induce apoptosis in MDA-MB-231 human breast cancer cells. On one hand, ursolic acid decreased mitochondrial membrane potential (ΔΨm), release of cytochrome, lowered Bcl-2/Bax ratio and cleaved caspase-9. On the other hand, ursolic acid was found to induce the appearance of Fas receptor and cleavage of caspase-8, -3 and poly ADP ribose polymerase (PARP). Therefore, it could be suggested that ursolic acid stimulate apoptosis in MDA-MB-231 cells through both intrinsic pathway and extrinsic pathway [110]. Ursolic acid has also been reported to induce apoptosis in MCF-7 breast cancer cells through regulating activities of nuclear transcription factors glucocorticoid receptor (GR) and Activator Protein-1 (AP-1), PARP cleavage, and the expression of Bcl-2 protein [133].

Except for anti-proliferative effect, we also evaluated the effect of 2α-hydroxyursolic acid on apoptosis in MDA-MB-231 human breast cancer cells. The results of TUNEL assay showed a significant increase of apoptotic cells after the treatment of 2α-hydroxyursolic acid. A proposed mechanism would be 2α-hydroxyursolic acid suppressed TRAF2 expression, activated expression of ASK1, and then upregulated expression of phosphorylated p38 and p53, which was followed by the upregulation of the Bax/Bcl-2 ratio and induced of the cleaved caspase-3 [88].

Oleanolic acid and its derivatives also displays important roles in inducing

apoptosis in breast cancer cell lines. Wu et al. [134] demonstrated that SZC015 showed strongest inhibitory effect against MCF-7 breast cancer cells viability among oleanolic acid and other 3 synthetic oleanolic acid derivative, namely SZC014, 009, 013. In addition, SZC015 was able to induce cell apoptosis through down-regulating caspase3, caspase9, release of cytochrome C, cleavage of PARP and increasing ratio of Bax/Bcl-2. The decrease of PI3K expression, p-IκBα/IκBα ratio, p65 protein level both in cytoplasm and nucleus, ratios of p-p38a/p38a, p-JNK1/JNK1, p-ERK1/2/ERK1/2 and significant increase of p-Akt/Akt ratio were observed after treating the MCF-7 cells with SZC015. These results may support the participation of MAPKs and PI3K/Akt/mTOR/NF-κB in SZC015's anti-cancer effect toward MCF-7 breast cancer cells [134]. According to a study conducted by the same group, SZC017, another synthetic oleanolic acid derivative, significantly decreased the cell viability of both MCF-7 and MDA-MB-231 breast cancer cell lines, but showed less toxic to MCF-10A mammary epithelial cell line. Along with decreased expression of both procaspase-9 and procaspase-3 and increased ratio of Bax/Bcl-2, SZC017 treatment suppressed the levels of Akt, p-Akt, p-IκBα, total p65 and p-p65 in both the cytoplasm and nucleus in MCF-7 cells. Furthermore, SZC017 treatment inhibited the p65 nuclear translocation. Taken together, SZC017 induced intrinsic apoptosis in MCF-7 breast cancer cell line. Rabi et al. [135] reported that Methyl 25-Hydroxy-3-oxoolean-12-en-28-oate (AMR-Me) isolated from *Amoora rohituka* stem bark exhibited pro-apoptotic effect through intrinsic mitochondrial apoptotic pathway in MCF-7 cells [135]. To illustrate the underlying mechanism, their recent research showed that AMR-Me significantly induced apoptosis proven by increased enrichment factor as well as

cleaved caspase-9 and PARP in MCF-7 cells and cleaved caspase-3 and PARP in MDA-MB-231 cells. In addition, AMR-Me downregulated ERα, PI3K p85, Akt1, and p-Akt in MCF-7 cells, decreased NF-κB DNA binding activity and abrogated EGF induced NF-κB activation in MDA-MB-231 cells, which indicated the involvement of these components in AMR-Me induced apoptosis [136]. HIMOXOL, another synthetic derivative of oleanolic acid, activate the extrinsic apoptotic pathway evidenced by activation of caspase-8, caspase-3 and PARP-1. The involvement of p38, JNK MAPK as well as NF-κB/p53 signaling pathways were detected [125].

Collectively, ursolic acid, 2α-hydroxyursolic, oleanolic acid and their derivatives have the potential to induce cancer cell apoptosis in various cancer cell lines through modulating associated molecular targets in intrinsic and extrinsic pathways.

1.4.1.3 Effect on the inhibition of angiogenesis and metastasis inhibition

Angiogenesis is the process of blood vessel formation from the existing vasculature. Angiogenesis provides a mode of transportation for primary cancer to become metastatic. Angiogenic factors, such as vascular endothelial growth factor (VEGF) and transforming growth factor β1 (TGF-β1), could stimulate endothelial cells nearby to multiply and lead to the formation of new capillaries [137, 138]. At the same time, metastasis takes place. Carcinoma cells become motile, invasive and migrate through the basement membrane. Urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs) play important roles in this process. Once activated by uPA, matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, could catalyze

the destruction of extracellular matrix (ECM) for cells to move [138, 139].

Huang et al. [140] demonstrated that ursolic acid inhibited interleukin-1 β (IL-1β) or tumor necrosis-α (TNF-α)-induced cell invasion in rat C6 glioma cells by transwell assay along with the suppression of MMP-9 expression and activity. The activations of upper stream regulator including protein kinase C- ξ (PKC- ξ) and NF-κB were also reduced by ursolic acid. Ursolic acid was also found to inhibit cell invasion and migration in both DU145 and PC-3 prostate cancer cells via downregulating CXCR4/CXCL12 signaling pathway [141]. The studies in Hep3B, Huh7 and HA22T liver cancer cells revealed that both ursolic acid and oleanolic acid diminished invasion and migration through reducing mRNA expression of VEGF, interleukin-8 (IL-8), uPA and hypoxia-inducible factor-1α (HIF-1α), which is crucial angiogenesis activator via lowering reactive oxygen species (ROS) and nitric oxide (NO) level [142]. In another study conducted by the same lab, ursolic acid also inhibited cell migration and invasion in A549, H3255 and Calu-6 lung cancer cells through suppressing expression of fibronectin, intercellular adhesion molecule-1 (ICAM-1), MMP-2 and MMP-9 and reducing protein kinase C (PKC) activity [143]. Similar effect was observed in another liver cancer cell line, Huh7 hepatocellular carcinoma cells. 2α-Hydroxyursolic acid significantly diminished cell migration by inhibiting VEGFR2/Src/FAK/cdc42 pathway [144]. Kim et al. [145] demonstrated that ursolic acid inhibited the invasive phenotype of SNU-484 gastric cancer cells by downregulating MMP-2 expression, while the expression of MMP-9 was not significantly reduced. US597, a novel ursolic acid derivative, was reported to inhibited cell adhesion, invasion and migration in HepG2 human liver cancer cells [146]. In

addition, US597 inhibit integrin-mediated focal adhesion signaling pathway through suppressing the expression of integrin α6, integrin β1 and downstream FAK, Src and paxillin, up-regulated expression of PTEN in HepG2 cells.

Approximately 50% of women with breast cancer will develop metastatic cancer, which remains the predominant the cause of death in patients with breast cancer [147-149]. Limited research have been done regarding the effect of triterpenoids on breast cancer metastasis. Yeh et al. [150] first observed an inhibitory effect of ursolic acid on the migration and invasion of MDA-MB-231 cells at non-cytotoxic concentrations. Ursolic acid suppressed mRNA levels of MMP-2 and uPA and their activities, as well as the expression of VEGF, while increased expression of tissue inhibitor of MMP-2 and plasminogen activator inhibitor-1, respectively. Ursolic acid also strongly reduced the levels of transcriptional factors including NF-κB p65, c-Jun and c-Fos in the nucleus of MDA-MB-231 cells. Phosphorylation of Jun N-terminal kinase, Akt and mammalian target of rapamycin, but not the phosphorylation of ERK and p38 were down-regulated by ursolic acid. The inhibition of upstream protein, such as anti-Rho-like GTPases (RhoA), anti- growth factor receptor-bound protein 2 (Grb2) and Ras were also observed [150]. Oleanolic acid isolated from Terminalia bentzoe L. leaves along with 13 derived semisynthetic analogues were tested for the ability to inhibit MDA-MB-231 cells migration, proliferation, and invasion. Among them, 3-O-[N-(3'-chlorobenzenesulfonyl)-carbamoyl]-oleanolic acid and 3-O-[N-(5'-fluorobenzenesulfonyl)-carbamoyl]-oleanolic acid were the most effective compounds in inhibiting migration and proliferation in MDA-MB-231 cells. This effect might be related, at least in part, to the suppression of Breast tumor kinase

(Brk)/Paxillin/ Ras-related C3 botulinum toxin substrate 1 (Rac1) signaling pathway [151].

Despite recent advances in tumor detection and treatment, metastasis remains the predominant cause of death in patients with breast cancer. Comprehensive research are required to thoroughly understand the effects of triterpenoids on breast cancer metastasis.

1.4.2 In vivo

In vitro studies have shown that ursolic acid, 2α-hydroxyursolic, oleanolic acid, and their derivatives exhibit strong inhibitory effect in various cancer cell lines including breast cancer cell lines. *In vivo* studies are still necessary to verify the effect of these triterpenoids in the prevention or treatment of breast cancer. Both chemically-induced and immunocompromised tumor growth were utilized in the following mouse or rat models to measure the efficacy of triterpenoids.

Ursolic acid, 2α-hydroxyursolic, oleanolic acid, and their derivatives have been reported to have anti-tumor effect in various animal models of cancer. According to a study using H22 xenograft model, anti-proliferative effect was observed in the group treated with 2.53 mg/mouse/day ursolic acid for 10 days [109]. Treatment with ursolic acid also induced apoptosis of tumor cells in mouse xenograft model accompanied with the elevation of caspase-3 and -8 expression. Ursolic acid has also been reported to decrease tumor growth in mice xenografted with HCT15 p53 mutant apoptosis-resistant colorectal carcinomas cells, possibly involving JNK signaling pathway [112].

In a colorectal cancer xenograft mice model, injected with 12.5 mg/kg ursolic acid resulted in less tumor volume and less tumor weight without changing body weight [69]. Saraswati et al. [152] investigated anti-cancer activity of ursolic acid in Ehrlich ascites carcinoma tumor in Swiss albino mice. The results showed that ursolic acid administration prolonged the survival, inhibited tumor growth and tumor-induced neo-vascularization of tumor bearing mice. In addition, ursolic acid suppressed VEGF, iNOS, TNF-α, increased IL-12 levels and induced apoptosis by triggering the activation of caspase-3, -9 and Bax. *In vivo* study conducted by Prasad et al. [153] in an orthotopic nude mouse model showed that ursolic acid (250 mg/kg once daily, orally) exhibit its anti-colorectal cancer effect through inhibiting tumor volume, ascites formation, and distant organ metastasis, this effect was enhanced when combined with capecitabine (60 mg/kg, twice weekly by gavage). Ursolic acid suppressed EGFR, transcription factors (NF-κB, STAT3, and β-catenin) and induced p53 and p21 expression. Expression of biomarkers of proliferation (Ki-67 and cyclin D1), microvessel density (CD31) and metastatic (MMP-9, VEGF, and ICAM-1) were inhibited. Prasad et al. [80] also reported that ursolic acid exhibited anti-cancer effect in an orthotopic pancreatic nude mouse model. Oral administration of 250 mg/kg ursolic acid inhibited tumor growth and the metastasis of cancer cells to distant organs such as liver and spleen. Immuno-histochemistry analysis of tumor tissue indicated that ursolic acid down-regulated Ki-67 and CD31. Ursolic acid inhibited the activation of NF-κB and STAT3, which further resulted in the inhibition of expression for cell survival (survivin and Bcl-2), proliferation (cyclin D1), inflammation (COX-2), and metastasis (ICAM-1 and MMP-9) in tumors. When ursolic acid was combined with 25

mg/kg gemcitabine, it proves to be more effective as an anti-cancer agent. More recently, *in vivo* chorioallantoic membrane (CAM) assay and H22 hepatoma xenograft mouse model were used to investigate the anti-cancer effect of ursolic acid-loaded chitosan nanoparticles (CH-UA-NPs) [154]. CH-UA-NPs effectively inhibited vessel formation of fertilized eggs and H22 tumor growth by blocking VEGF signaling pathway.

2α-Hydroxyursolic acid also exhibit strong anti-cancer effect *in vivo*. The antitumor effect of 2α-hydroxyursolic acid was first tested in a mouse model of osteosarcoma by Horlad et al [155]. Oral administration of 17.5 mg/kg 2α-hydroxyursolic acid before and after subcutaneous implantation with LM85 murine osteosarcoma cells in mice significantly inhibited both subcutaneous tumor development and lung metastasis without suppressing tumor proliferation index. In addition, 2α-hydroxyursolic acid reversed the immunosuppressive activity of myeloid-derived suppressor cells (MDSC), which suppress of T-cell activation. 2α-Hydroxyursolic acid also displayed significant anti-tumor activity in a hepatocellular carcinoma xenograft mice model [144]. Huh7 hepatocellular carcinoma cells (2 × 106 cells) were injected subcutaneously into the flanks of each mouse. After one week, the mice were treated with 50 μL DMSO (control) or 2α-hydroxyursolic acid (5 mg/kg/day) by intraperitoneal injection for 21 days. The results showed that CA reduced tumor volume and mass with little toxic effects accompanied with the inhibition of phosphorylation of both VEGFR2 and FAK in xenograft mice.

De Angel et al. [156] investigated the effect of ursolic acid on breast cancer in an animal model. The 6-week-old ovariectomized female C57BL/6 mice were

randomized to receive control diet (AIN-93G) or diet supplemented with ursolic acid at 1 of 3 doses (0.05%, 0.10%, or 0.25% wt/wt in the diet). After 3 weeks on the diet treatments, mice were injected with syngeneic MMTV-Wnt-1 mammary tumor cells in the mammary fat pad; the respective diets were continued for 5 additional weeks. Interestingly, the mice that were fed with 0.10 % ursolic acid dose (≈106 mg/kg body weight/day) had significantly smaller tumors than control throughout the study. In addition, all the doses decreased tumor cell proliferation. The underling mechanism of anti-tumor effect of ursolic acid might be the regulation of Akt phosphorylation, pS6, cleaved-caspase-3, Cyclin D1 in tumor tissue. The growth inhibitory effects of ursolic acid on WA4 mammary tumor cells were also tested to further elucidate the effect of ursolic acid on mammary tumor size. Ursolic acid inhibited cell proliferation, suppressed colony formation and caused G1 arrest in WA4 cells.

The synthetic triterpenoid CDDO-Im, the Gemini vitamin D analog BXL0124 and the combination on the prevention and treatment of mammary tumor were investigated [157]. Preventative experiments were conducted such that female MMTV-ErbB2/neu transgenic mice (6–7 weeks old) were orally administrated with CDDO-Im (3 μmol/kg body weight), BXL0124 (0.3 μg/kg body weight), or the combination three times a week from three months of age until the end of the experiment. CDDO-Im and the combination treatments showed significant effect in reducing average tumor burden as compared to control without showing toxicity. All three treatments decreased the activation of ErbB2 (HER2) signaling pathway, including ErbB2 itself, activated-Erk1/2, activated-Akt, c-Myc, Cyclin D1, and Bcl2. Results indicated that the combination of CDDO-Im and BXL0124 was given to

MMTV-ErbB2/neu mice mammary tumors were established between 23 and 30 weeks of age. Neither the effects on tumor growth nor the ErbB2 signaling pathway was observed by the short-term treatment [157]. CDDO-Me, another synthetic triterpenoid, played a potential role in the breast cancer–associated gene 1 (*BRCA1*) muted breast cancer prevention. Since 12 weeks old, $Brca1^{Co/Co}$; MMTV-Cre; $p53^{+/-}$mice were fed control diet or a diet containing CDDO-Me (50 mg/kg diet). CDDO-Me significantly delayed tumor development, reduced the average number of tumors per mice and average tumor burden, increased average lifespan. Immunohistochemical analysis showed reduced level of ErbB2, p-ErbB2, and Cyclin D1 in the mammary glands when treated with CDDO-Me. Data from *in vitro* study indicated that CDDO-Me inhibited progression through the cell cycle and reduced phosphorylation of ErbB2, which verified the conclusion drew from animal study [158]. This group also investigated the anti-tumor effect of CDDO-Me in a polyoma virus middle T oncoprotein (PyMT) mouse model. Four-week old female PyMT mice were fed control diet or CDDO-Me (50 mg/kg diet). CDDO-Me treatment delayed the tumor development as well as expanded the lifespan compared to control group. However, no effect on tumor number or tumor size was observed in this study. *In vitro* study conducted in primary tumor cells from PyMT mice showed that CDDO-Me decreased proliferation of these cells by inhibiting Cyclin D1 and reducing phosphorylation of EGFR and STAT3. MMP-9 secretion was also significantly decreased by CDDO-Me [159]. Methyl 25-Hydroxy-3-oxoolean-12-en-28-oate (AMR-Me) has been reported to exhibit remarkable inhibitory effect against 7, 12-dimethylbenz(a)anthracene

(DMBA)-initiated rat mammary tumor in an experimental rodent tumor model. Pathogen-free virgin female Sprague-Dawley rats were orally administered with different dosed of AMR-Me (0.8, 1.2 and 1.6 mg/kg) three times per week for two weeks. Then mammary carcinogenesis was initiated by oral administration of DMBA (50 mg/kg body weight). AMR-Me treatment lasted another 16 weeks, DMBA-induced mammary tumor incidence, total tumor burden and average tumor weight were suppressed without toxicity. AMR-Me inhibited cell proliferation, induced apoptosis, up-regulated Bax and down-regulated Bcl-2, PCNA protein expression in mammary tumors. RT-PCR analysis showed up-regulation of Bax, Bad, caspase 3, caspase 7 and PARP mRNA level and down-regulation of Bcl-2 mRNA level, which explained the pro-apoptotic mechanisms involved in AMR-Me-mediated prevention of mammary tumorigenesis [160].

Overall, both *in vitro* and *in vivo* findings suggest that ursolic acid, 2α-hydroxyursolic, oleanolic acid and their derivatives exert anti-proliferative, pro-apoptotic and anti-metastatic effects in various cancer cell lines including breast cancer cell lines, which makes them good pharmaceutical candidates and helps to understand the health benefits of fruits and vegetables.

1.5 Objective

As the most common cancer diagnosed among women in the United States, breast cancer accounts for nearly one third of all cancers in women [162]. MDA-MB-231 breast cancers are triple-negative breast cancer, which has limited number of receptors and lacks an established therapeutic target [5]. Epidemiological studies showed

increased consumption of whole grains, vegetables, and fruits has been associated with reduced risk of breast cancer [19-25]. Fruits, vegetables, and other plant-based foods are rich in bioactive phytochemicals that might account for the inhibitory effects in the stages of promotion and progression of carcinogenesis [163, 164]. 2α-Hydroxyursolic acid, as a member of phytochemical family, has been isolated from apple peels [165]. It was also reported that 2α-hydroxyursolic acid displayed anti-cancer effect against several human cancer cell lines [87, 115, 119, 130]. However, the underlying mechanisms of actions of 2α-hydroxyursolic acid in the prevention of cancer were not fully understood. In addition, invasion and metastasis constitute 90% of cancer related mortality. The inhibition of cancer cell metastasis has become an attractive alternative therapeutic approach to alleviate cancer resulted death. Therefore, the goal of this study is to investigate the mechanism of 2α-hydroxyursolic acid in inhibiting cell proliferation, inducing of cell apoptosis and suppressing cell metastasis in human MDA-MB-231 breast cancer cells.

1.5.1 Hypotheses

Hypothesis: 2α-Hydroxyursolic acid may have anti-cancer activities in regulating proliferation, apoptosis and metastasis in MDA-MB-231 human breast cancer cells through targeting the signaling transduction pathway.

1.5.2 Objectives

To meet the goal and verify the hypothesis, I propose to accomplish the following

objectives:

Objective 1: To determine if 2α-hydroxyursolic acid inhibit proliferation and induce apoptosis in MDA-MB-231 human breast cancer cells through p38/MAPK signal transduction pathway.

Objective 2: To determine if 2α-hydroxyursolic acid inhibit HGF-induced metastasis in MDA-MB-231 human breast cancer cells though HGF/Met/Akt signal transduction pathway.

Objective 3: To determine if 2α-hydroxyursolic acid inhibit EGF-induced metastasis in MDA-MB-231 human breast cancer cells through EGF/EGFR/Akt signal transduction pathway.

1.6 Implications and future research

Our study demonstrated that treatment of MDA-MB-231 human breast cancer cells with 2α-hydroxyursolic acid results in significant inhibition of cell proliferation and the induction of apoptosis through p38 MAPK signaling pathway, inhibition of cell metastasis through both HGF/Met-PI3K/Akt-NF-κB and EGF/EGFR-PI3K/Akt-NF-κB/AP-1 signal transduction pathways. Our findings suggested that consumption of fruits and vegetables with bioactive 2α-hydroxyursolic acid might be beneficial to prevent or coordinate with treatment of triple-negative breast cancer. In further studies, the involvement of other alternative pathways in 2α-hydroxyursolic acid inhibited metastasis needs investigation. In addition, additive and synergistic interactions of phytochemicals present in whole food are likely to display better effect against chronic disease including breast cancer based on accumulative evidence. Therefore, more

efforts should be devoted to explore the potential of these multifunctional agents in combinational settings. Animal models and clinical studies are required to verify the efficacy of these agents as potential candidates for breast cancer prevention and treatment.

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CHAPTER 2

2α -HYDROXYURSOLIC ACID INHIBITED CELL PROLIFERATION AND INDUCED APOPTOSIS IN MDA-MB-231 HUMAN BREAST CANCER CELLS THROUGH P38/MAPK SIGNAL TRANSDUCTION PATHWAY

Abstract

The mechanisms of action of 2α-hydroxyursolic acid in inhibiting cell proliferation and inducing apoptosis in MDA-MB-231 human breast cancer cells were investigated. The anti-proliferative activity and cytotoxicity were determined by the methylene blue assay. The expression of proteins was determined using Western blot. 2α-Hydroxyursolic acid significantly inhibited MDA-MB-231 cells proliferation and no cytotoxicity was observed at the concentration below 30 μM. 2α-Hydroxyursolic acid significantly down-regulated expression of TRAF2, PCNA, Cyclin D1, CDK4 and up-regulated the expression of p-ASK1, p-p38, p-p53, p-21. 2α-Hydroxyursolic acid induced apoptosis in MDA-MB-231 cells by significantly increasing the Bax/ Bcl-2 ratio and inducing the cleaved caspase-3. Additionally, treatment of SB203580, p38 MAPK specific inhibitor, reversed the inhibition of PCNA, Cyclin D1 and Bcl-2 expression induced by 2α-hydroxyursolic acid in MDA-MB-231 cells. These results suggested that 2α-hydroxyursolic acid exhibited anti-cancer activity through the inhibition of cell proliferation and induction of apoptosis by regulating p38/MAPK signal transduction pathway.

Key Words: 2α-hydroxyursolic acid, apoptosis, breast cancer, cell proliferation, diet and cancer, phytochemicals

2.1 Introduction

Cancer is a major health problem not only in the United States, but also worldwide. One in four deaths in the United States is due to cancer [1]. As the most common cancer diagnosed in the United States, breast cancer accounts for nearly one third of all cancers in women [2]. Cancer is usually treated by surgery, radiation, chemotherapy, targeted therapy, and immunotherapy. Triple-negative breast cancer accounts for approximately 15–20 % of all breast cancer, and is defined as tumors that are estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and human epidermal growth factor receptor 2 (HER2)-negative [3, 4]. Unlike other subtypes, triple-negative breast cancer lacks an established therapeutic target, which leads to less progress in the treatment. In line with the side effects and the strong pain patients suffered, there is an urgent need for development of new treatment approaches [5].

It is estimated that about 30% of all cancers have been attributed to diet [6], which suggested that the changes in dietary behavior, such as increasing consumption of fruits, vegetables, and whole grains are practical strategies to reduce the incidence of cancers and other chronic diseases [7]. Epidemiological studies showed that increased consumption of whole grains, vegetables, and fruits have provided a significant inverse association with breast cancer [8-14]. Fruits, vegetables, whole grains, and other plant-based foods are rich in bioactive phytochemicals that might account for the inhibitory effects in the stages of promotion and progression of

carcinogenesis [15, 16]. Dietary phytochemicals have been demonstrated to have complementary mechanisms of actions for cancer prevention, including scavenging free radicals, deactivation and removal of carcinogens, modulation of detoxification phase II enzymes, DNA damage repair, inhibition of cell proliferation, regulation of cell cycle and gene expression through signal transduction pathways, induction of apoptosis, inhibition of nuclear factor κB activation, anti-inflammation, anti-angiogenesis, stimulation of the immune system, regulation of hormone metabolism and receptors, and antibacterial and antiviral effects [15, 17, 18].

Apples are an important part of the diet in human and are largest supplier of fruit phenolics (33%) in the American diet [19]. Apple peels, which are often discarded, have a high content of phytochemicals, which might contribute to antioxidant activity and anti-proliferative activity to provide health benefits when consumed [20-22]. 2α-Hydroxyursolic acid, also named corosolic acid, has been isolated from apple peels with high anti-proliferative activity previously [23]. Despite the presence of 2α-hydroxyursolic acid in fruits and vegetables including fruits of *Crataegus pinnatifida var. psilosa* [24], *Hippophae rhamnoides* [25], *Mours alba L.* (mulberry), *Ocimum basilicum* (basil), *Toona sinensis* (mahogany) and *Hemerocallis fulva L.* (daylily) [26], 2α-hydroxyursolic acid has also been discovered in various traditional Chinese medicinal herbs. These include *Lagerstroemia speciosa* [27], *Eriobotrta japonica* [28], *Tiarella polyphyll* [29], and *Potentilla discolor Bunge* [30]. Previous research reported that 2α-hydroxyursolic acid exhibits a variety of pharmacological effects including anti-diabetic activity [27, 31-33], anti-inflammation [34], anti-obesity [28, 35], anti-atherosclerosis [36], and osteoblast differentiation

stimulating activity [37]. It was also reported that 2α-hydroxyursolic acid displayed anti-cancer effect against several human cancer cell lines [23, 38-41]. However, the mechanisms of action for 2α-hydroxyursolic acid against breast cancer were not fully understood. The objective of this study was to investigate the mechanisms of action of 2α-hydroxyursolic acid in inhibiting cell proliferation and inducing apoptosis in a triple-negative breast cancer cell line, MDA-MB-231 human breast cancer cells.

2..2 Materials and Methods

2.2.1 Chemicals

2α-Hydroxyursolic acid was isolated from apple peels by our lab as described previously [23]. Dimethyl sulfoxide (DMSO) was purchased from VWR (Radnor, PA, USA). Phosphate-buffered saline (PBS) and α-Minimum Essential Medium (α-MEM) were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). Polyoxyethylene (tween 20) sorbitan monolaurate was obtained from Calbiochem (Billerica, MA, USA). Hepes, methyl green and protease inhibitors (aprotinin; leupeptin; pepstain; sodium orthovanadate) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.2.2 Antibodies

Primary antibody against PCNA was obtained from Calbiochem (Billerica, MA). Antibodies against CDK-4, Cyclin D1, p21, cleaved caspase 3, Bcl-2, Bax, p-p53, p-

ASK-1 and TRAF-2 were purchased from Santa Cruz Biotechnolgy, Inc. (Santa Cruz, CA). P-p38 was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against β-actin and α-tubulin, anti-mouse IgG and anti-rabbit IgG were obtained from Sigma-Aldrich Inc. (St. Louis, MO).

2.2.3 Cell culture

MDA-MB-231 human breast cancer cells purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained at 37 °C in a humidified 5% CO_2 atmosphere in α-MEM containing 10 mM Hepes, 1% antibiotic-antimycotic and 10% heat-inactivated fetal bovine serum as described previously [41, 42].

2.2.4 Assessment of cytotoxicity by methylene blue assay

2α-Hydroxyursolic acid was dissolved in Dimethyl sulfoxide (DMSO) and diluted to working concentrations with growth medium. The cytotoxicity of 2α-hydroxyursolic acid toward MDA-MB-231 human breast cancer cells was measured by methylene blue assay as reported previously by our laboratory [43]. MDA-MB-231 cells in 100 µL fresh medium were plated in 96-well plate at a density of 4.0×10^4 cells/well and were incubated at 37 °C in 5% CO_2 for 24 h. Then, the growth medium was removed and the cells were treated with different concentrations of 2α-hydroxyursolic acid as samples or only 1% DMSO as control in 100 µL fresh medium. After another 24 hours incubation, the growth medium was removed from each well and the cells were washed with 100 µL phosphate-buffered saline (PBS). Cells were then stained with

methylene blue solution [98% Hanks Balanced Salt Solution (HBSS), 0.67% glutaraldehyde, 0.6% methylene blue] and incubated for 1 hour. After that, the solution was removed and rinsed with deionized water three times. After the wells were air-dry, methylene blue stain in cells was eluted with the elution buffer [1% (v/v) acetic acid, 49% (v/v) PBS, and 50% (v/v) ethanol] by rotating on a bench shaker for 20 minutes. The absorbance was read at 570 nm by using FilterMax F5 Multi-Mode Microplate Readers (Molecular Devices, Sunnyvale, CA). Cytotoxicity was determined as percentage compared to the control. All measurements were conducted in triplicate.

2.2.5 Assessment of anti-proliferative activity by methylene blue assay

The anti-proliferative activity of 2α-hydroxyursolic acid was measured by the methylene blue assay described previously [43]. MDA-MB-231 human breast cancer cells in 100 µL fresh medium were plated in 96-well plate at a density of 2.5×10^4 cells/ well and were incubated at 37 °C in 5% CO_2 for 8 h. Then, the growth medium was removed and the cells were treated with different concentrations of 2α-hydroxyursolic acid as samples or 1% DMSO only as control in 100 µL fresh medium. After additional 72 hours incubation, the growth medium was removed from each well and the cells were washed with 100 µL PBS buffer. Cells were stained and counted using methylene blue as described above. The anti-proliferative activity was determined as percentage compared to the control. All measurements were conducted in triplicate.

2.2.6 Determination of cell apoptosis

Cell apoptosis was measured in situ using the ApopTag@Plus Peroidase In Situ Apoptosis Detection Kit based on the terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling (TUNEL) assay as reported previously [44]. Briefly, 3×10^5 cells were seeded on a Falcon 8-chamber culture slide. After reaching 60–70% confluence, the cells were starved with serum free medium for 2 hours. The cells were then treated with different concentrations of 2α-hydroxyursolic acid for 4 h. After washed with PBS, the cells were then fixed in 1% paraformaldehyde for 20 min at room temperature, and post-fixed in pre-cooled ethanol:acetic acid (2:1, v:v) at -20 °C for another 5 min. 3% H_2O_2 was used to quench endogenous peroxidase for 15 min at room temperature. Cell DNA fragments were labeled with peroxidase, which were then reacted with the peroxidase substrate, 3, 3'-diaminobenzidine (DAB), to give a permanent, localized brown-color stain. Methyl green was used to stain the normal cells to further differentiae from apoptotic cells. Finally, 2000 cells from each treatment were randomly selected for observation. Results were expressed as the percentage of apoptotic cells (mean ± SD) and conducted in triplicate.

2.2.7 Western blot assay

The western blot analysis was carried out as described previously [21, 45]. Briefly, MDA-MB-231 human breast cancer cells were seeded at a density of 5.0×10^5 cells/ well in 6-well plates for 8 hours and then treated with various concentrations of 2α-

hydroxyursolic acid for another 24 hours. Then the cells were washed twice with ice-cold PBS and scraped off from the wells. Harvested cells were then lysed using lysis buffer (50 mM Tris, pH 7.4; 1% Igepal; 150 mM sodium chloride; 1 mM EDTA) with different protease inhibitors (1 g/mL aprotinin; 1 g/mL leupeptin; 1 g/mL pepstain; 1 mM sodium orthovanadate). Cell lysates were vortexed briefly every 5 min for half an hour to facilitate protein extraction. Lysates were then centrifuged at 12000 g for 5 min at 4 °C, and protein concentrations of the lysates were determined using a Sigma Diagnostics Micro Protein Determination Kit and a FilterMax F5 Multi-Mode Microplate Readers (Molecular Devices, Sunnyvale, CA). Equal amounts of protein from each cell lysate was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes [21, 45]. The membranes were blocked for 2 h with 5% non-fat dry milk in TBST (Tris-base buffer solution containing of 0.1% Tween 20) at room temperature and incubated with the desired primary antibody (all in 1:1,000 dilutions in 1% nonfat dry milk and TBST) overnight at 4˚C. After secondary antibody was applied, membrane-bound antibodies were visualized by the Enhanced Chemiluminescence kit (Cell Signaling Technology, Inc., Beverly, MA) according to the manufacturer's instruction. Bands were then scanned and quantified by ImageJ2x software (Wayne Rasband, National Institutes of Health, Maryland, USA). The expression of human α-tubulin or β-actin was used as an internal standard control. All measurements were conducted in triplicate.

2.2.8 Inhibitor treatment

MDA-MB-231 human breast cancer cells were pretreated with 0, 50 nM, 1 μM or 20 μM p38 MAP kinase specific inhibitor SB203580 for 1.5 h, and followed by treatment with or without 25 μM 2α-hydroxyursolic acid for another 24 h. The cells were collected and lysed for Western blot assay as described above.

2.2.9 Statistical analysis

Data were analyzed using Sigmaplot software version 11.0 (Systat Software, Inc. Chicago, IL) and dose-effect analysis was performed using Calcusyn software version 2.0 (Biosoft, Cambridge, UK). Data were presented as mean ± SD for at least three independently performed experiments. Statistical analyses were carried out with Student's t-test and analysis of variance (ANOVA) by JMP software version 9.0.2 (SAS Institute Inc. North Carolina, USA). Differences with $p<0.05$ were considered to be statistically significant.

2.3 Results

2.3.1 2*α*-Hydroxyursolic acid inhibits proliferation of MDA-MB-231 human breast cancer cells

It has been well known that cancer is caused by the dysregulated proliferation of cells [46]. Therefore, proliferation of MDA-MB-231 human breast cancer cells was examined to determine whether 2α-hydroxyursolic acid had inhibitory effect on growth of MDA-MB-231 human breast cancer cells. Cells were treated with various

concentrations of 2α-hydroxyursolic acid (0, 5, 10, 15, 20, 25, 30, 35, and 40 μM), which significantly inhibited proliferation of MDA-MB-231 human breast cancer cells at the doses of 15 μM and above in a dose-dependent manner (Figure 2.1, $p<0.05$). The median effective dose (EC_{50}) of 2α-hydroxyursolic acid for inhibition of MDA-MB-231 human breast cancer cell proliferation was 19.82 μM. The inhibitory effects were apparent at concentrations of 15, 20, and 25 μM of 2α-hydroxyursolic acid with 17, 57 and 68% inhibitory rates, respectively, while no cytotoxicity were observed at the concentrations lower than 30 μM under these conditions. Thus, these concentrations were selected for subsequent studies. However, the inhibition of proliferation of MDA-MB-231 human breast cancer cells by 2α-hydroxyursolic acid at 30 μM and above may be due to its cytotoxicity (Figure 2.1).

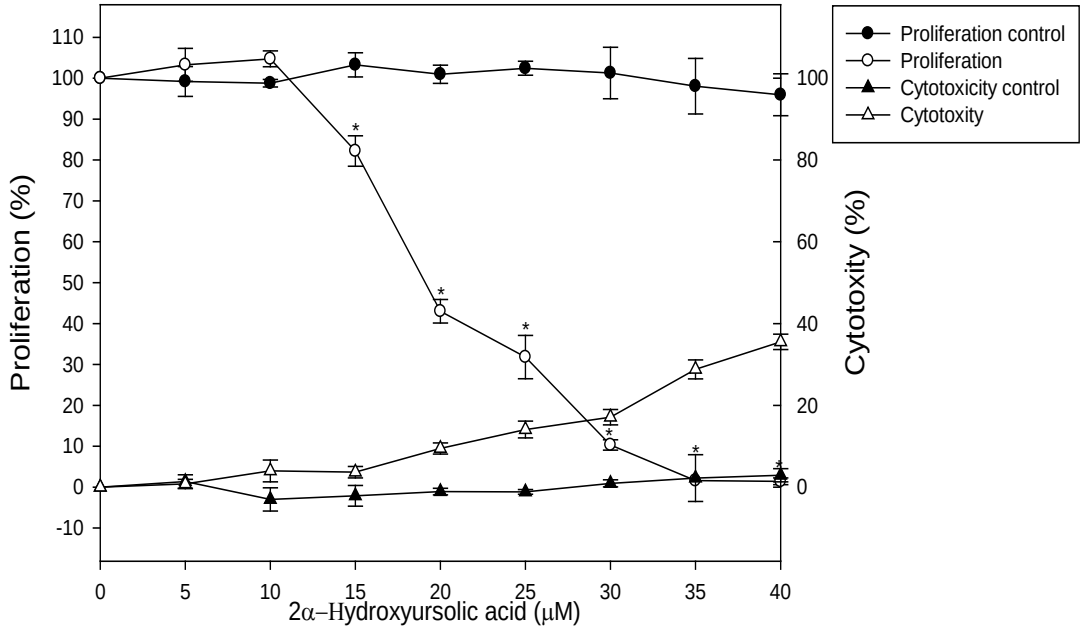


Figure 2.1. Effect of 2α-hydroxyursolic acid on cell proliferation and cytotoxicity in MDA-MB-231 human breast cancer cells. An asterisk (*) indicates a significant difference from the control ($p < 0.05$). Each value represents the mean ± SD of triplicates.

2.3.2 Effects of 2α-hydroxyursolic acid on the expression of proteins involved in the proliferation and cell cycle in MDA-MB-231 human breast cancer cells.

To confirm the anti-proliferative activity of 2α-hydroxyursolic acid towards MDA-MB-231 human breast cancer cells, the expression of proliferating cell nuclear antigen (PCNA) was measured. Western blotting analysis of cells treated with various concentrations of 2α-hydroxyursolic acid for 24 h indicated that the protein expression of PCNA was down-regulated in a dose-dependent manner (Figure 2.2A). In the group receiving 25 μM of 2α-hydroxyursolic acid, the PCNA expression was inhibited to 38.88% when compared to the control ($p<0.05$). Cyclin D1 and Cyclin-dependent kinases-4 (CDK4) are essential regulators that control the G1-S transition of the cell cycle [47]. Therefore, to further investigate the modulation of cell cycle proteins, we also examined the expression of p21, Cyclin D1 and CDK4 in the MDA-MB-231 human breast cancer cells after the treatment of 2α-hydroxyursolic acid at doses of 0, 15, 20, and 25 μM for 24h (Figure 2.2B-D). Data from Western blot analysis showed that 2α-hydroxyursolic acid significantly decreased the protein levels of pro-proliferative Cyclin D1 and CDK4 in MDA-MB-231 human breast cancer cells (Figure 2.2B and 2.2C, $p<0.05$). Meanwhile, the protein levels of anti-proliferative

p21, known as Cyclin-dependent kinase inhibitor, was significantly increased after 2α-hydroxyursolic acid treatment (Figure 2.2D, $p<0.05$).

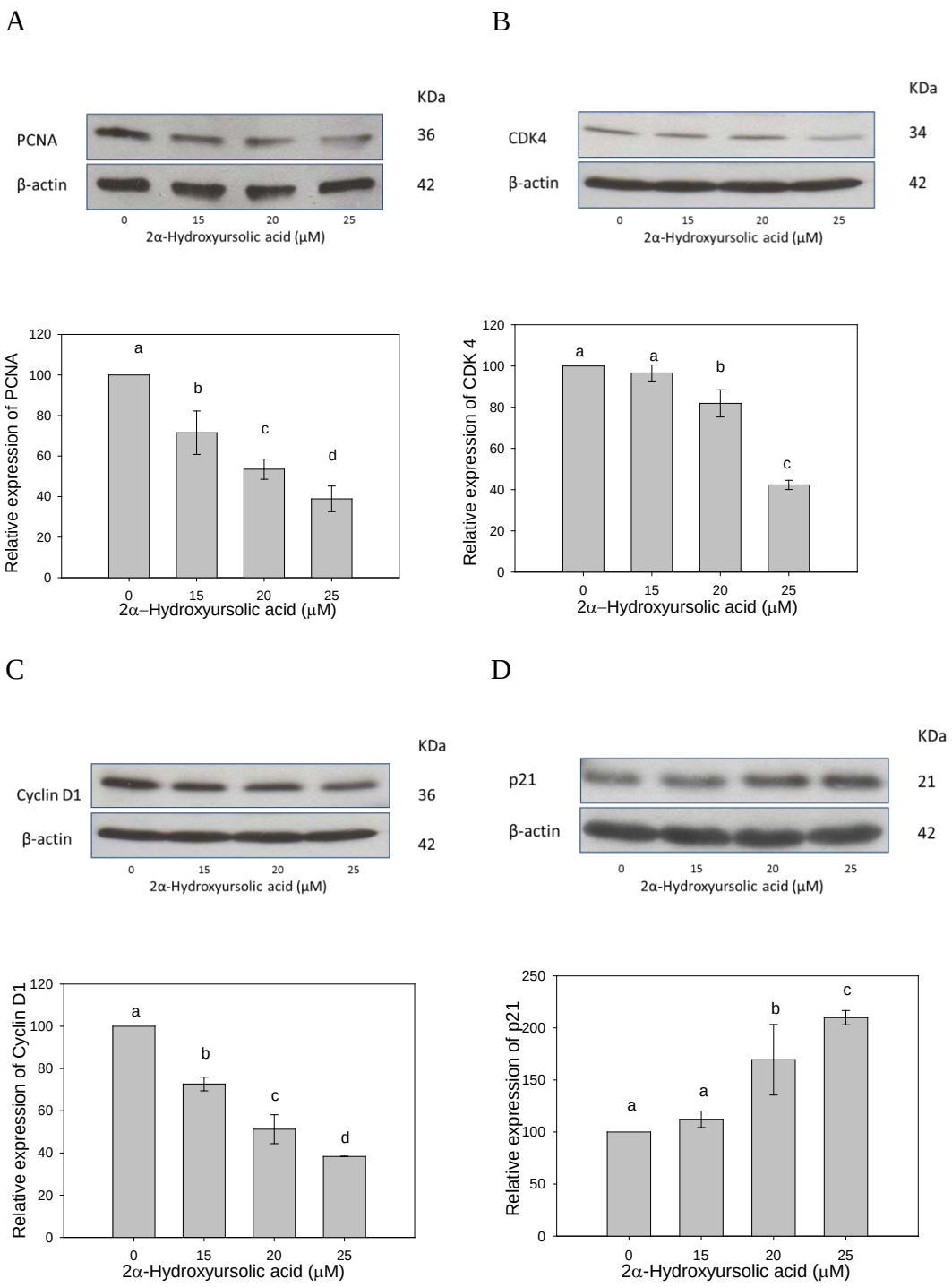


Figure 2.2. Effects of 2α-hydroxyursolic acid on expression of PCNA (A), CDK4 (B), Cyclin D1 (C) and p21 (D) in MDA-MB-231 human breast cancer cells. Bars with no

letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

2.3.3 p38 MAP kinase pathway has involved in 2α-hydroxyursolic acid regulated proliferation

Phosphorylated p53 (p-p53) was hypothesized to be involved in cell proliferation control induced by 2α-hydroxyursolic acid in MDA-MB-231 human breast cancer cells. As shown in Figure 2.3A, 2α-hydroxyursolic acid significantly up-regulated the expression of p-p53 in a dose-dependent manner in MDA-MB-231 human breast cancer cells when compared to the control (Figure 2.3A, $p<0.05$). p38 MAP kinase pathway has been shown to play a key role in the regulation of cell growth, apoptosis and cell cycle progression [48]. Our data showed that phosphorylated p38 (p-p38) protein expression was significantly increased at the doses of 20 and 25 μM (Figure 2.3B, $p<0.05$), indicating p-p38 was involved in 2α-hydroxyursolic acid-induced anti-proliferative activity in MDA-MB-231 human breast cancer cells. The effect of 2α-hydroxyursolic acid on upstream protein modulators, apoptosis signal-regulating kinase 1 (ASK1), an activator of p-p38, and TNF receptor associated factor 2 (TRAF2) (Figure 2.3C and 3D) have been examined. The expression of p-ASK1 in MDA-MB-231 human breast cancer cells was significantly increased in a dose-dependent manner after the treatment with 2α-hydroxyursolic acid when compared to the control (Figure 2.3C, $p<0.05$). On the contrary, 2α-hydroxyursolic acid

significantly down-regulated the protein expression of TRAF2, which functions as a mediator of the anti-apoptotic signal, as shown in Figure 2.3D.

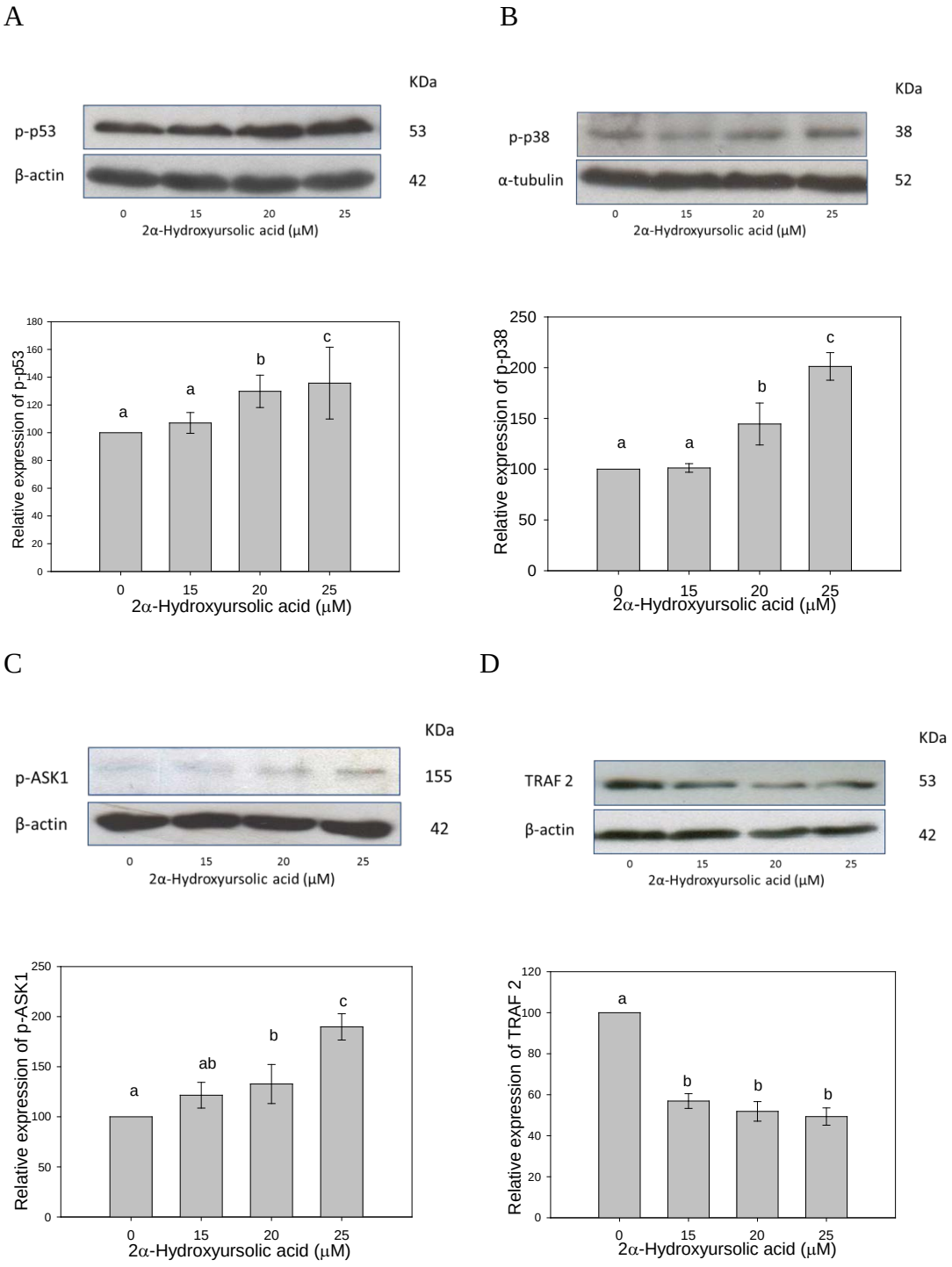


Figure 2.3. Effects of 2α-hydroxyursolic acid on expression of bcl2 (A), bax (B), bax/bcl2 (C) and cleaved caspase 3 (D) in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ±SD of triplicates.

2.3.4 2α-Hydroxyursolic acid induces apoptosis in MDA-MB-231 human breast cancer cells

We examined the effect of 2α-hydroxyursolic acid on apoptosis via TUNEL assay to evaluate the effect of 2α-hydroxyursolic acid on MDA-MB-231 human breast cancer cell growth. Cells treated with 2α-hydroxyursolic acid showed typical morphology for apoptosis: nuclear fragmentation, chromatin condensation, and loss of membrane asymmetry (data not shown). For control, the apoptotic cells only accounted for 3 out of 100 cells. In comparison, the apoptotic cells were significantly increased to 4.5, 7.4, and 9.2 per 100 cells after the treatment of 2α-hydroxyursolic acid at the concentrations of 15, 20, and 25 μM, respectively (Figure 2.4). Thus, treatment of 2α-hydroxyursolic acid significantly induced apoptosis in MDA-MB-231 human breast cancer cells in a dose-dependent manner (Figure 2.4, $p<0.05$).

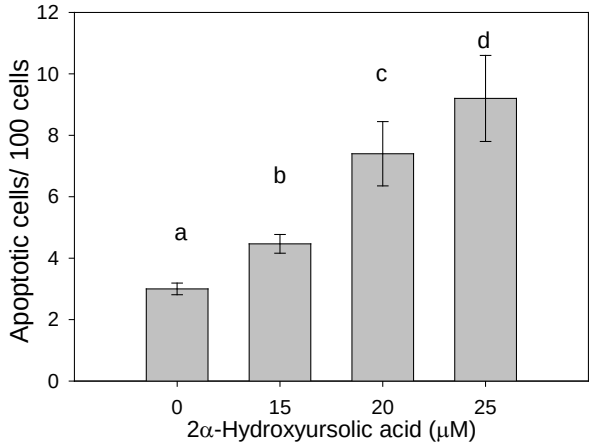


Figure 2.4. Induction of apoptosis of MDA-MB-231 human breast cancer cells by 2α-hydroxyursolic acid at the concentrations of 0, 15, 20 and 25 μM. Bars with no letters are in common significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

2.3.5 Effects of 2α-hydroxyursolic acid on the expression of proteins involved in the apoptosis in MDA-MB-231 human breast cancer cells.

To investigate the mechanisms of the pro-apoptotic activity of 2α-hydroxyursolic acid in MDA-MB-231 human breast cancer cells, expression of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and cleaved caspase-3 was evaluated by Western blotting analysis (Figure 2.5). It is well known that Bax, a pro-apoptotic protein and Bcl-2, an anti-apoptotic protein, are involved in cell apoptosis. As shown in figure 2.5B, 2α-hydroxyursolic acid significantly increased the protein expression of Bax

(1.52-, 1.81- and 2.11-fold vs. control for 15, 20, and 25 μM, respectively). In contrast, the protein expression of Bcl-2 was decreased by 2α-hydroxyursolic acid treatment (Figure 2.5A). Taken together, the results illustrated that 2α-hydroxyursolic acid induced a significant increase in the Bax/Bcl-2 ratio (Figure 2.5C). Furthermore, in an attempt to identify if the treatment of 2α-hydroxyursolic acid can change cleaved caspase-3 expression, a significant increase of cleaved caspase-3 was observed after the treatment of 2α-hydroxyursolic acid in a dose-dependent manner ($p<0.05$) (Figure 2.5D).

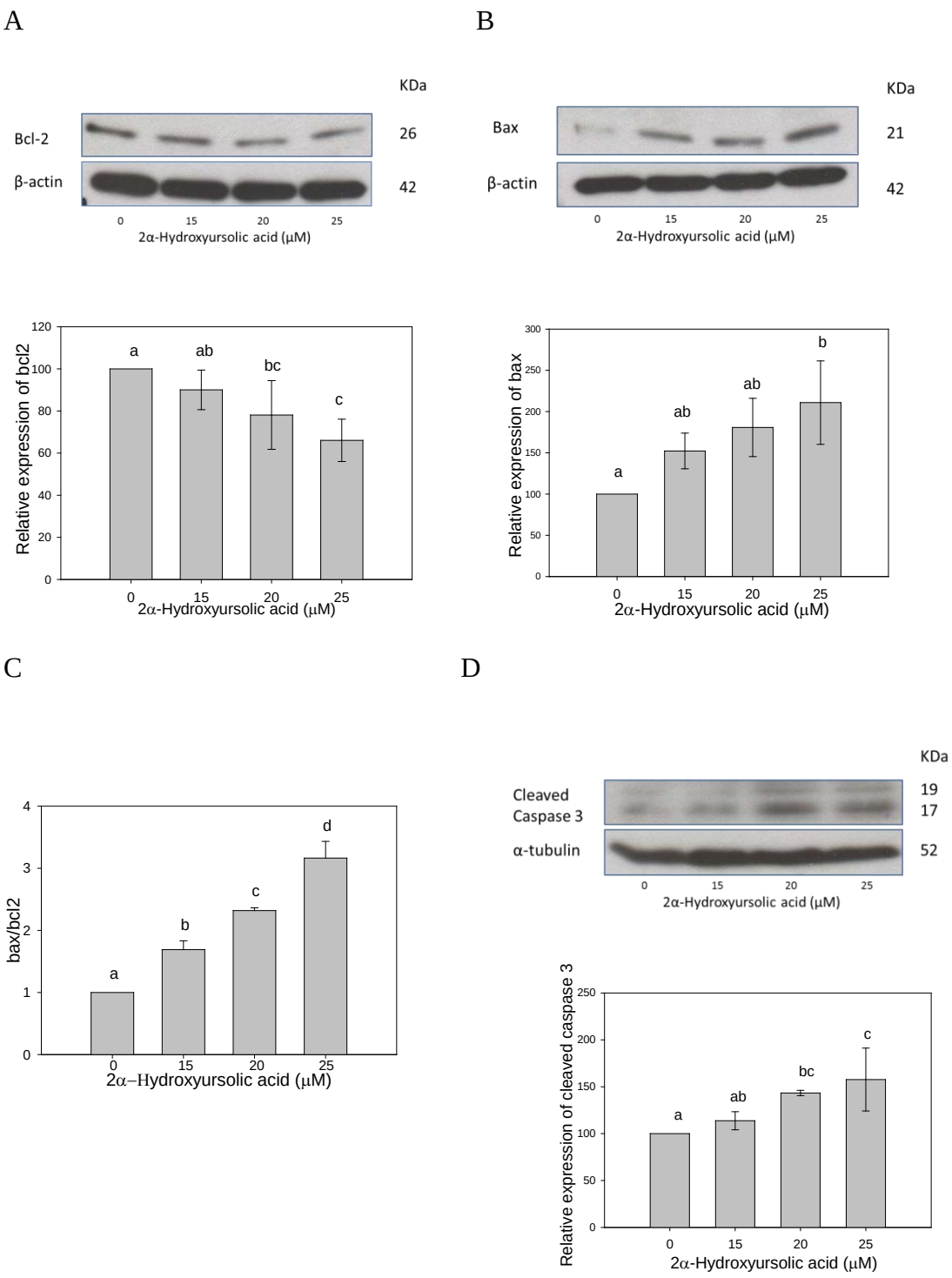


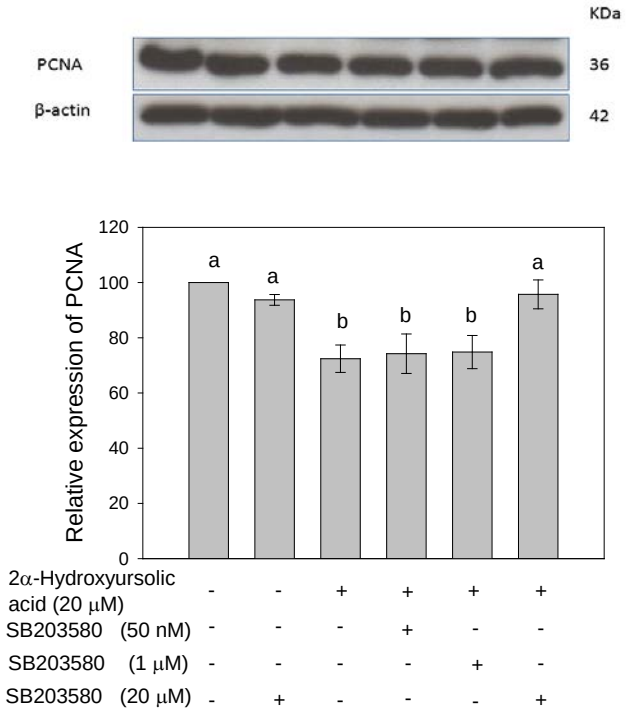
Figure 2.5. Effects of 2α-hydroxyursolic acid on expression of p-p53 (A), p-p38 (B), p-ASK1 (C), and TRAF 2 (D) in MDA-MB-231 human breast cancer cells. Bars with

no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

2.3.6 2α-Hydroxyursolic acid impacts the proliferation and apoptosis through p38 MAP kinase pathway

To elaborate the role of p38 MAP kinase in regulating cell proliferation and apoptotic activity, we tested the effect of p38 MAP kinase specific inhibitor, SB203580, on expression of PCNA, Cyclin D1 and anti-apoptotic protein Bcl-2 in MDA-MB-231 human breast cancer cells. MDA-MB-231 human breast cancer cells were pretreated with different concentrations (0, 50 nM, 1 μM or 20 μM) of p38 specific inhibitor SB203580 and followed by treatment with or without 25 μM 2α-hydroxyursolic acid. Pre-treatment of MDA-MB-231 cells with 20 μM SB203580 completely abolished 2α-hydroxyursolic acid-inhibited expression of PCNA, Cyclin D1 and Bcl-2, respectively (Figure 2.6A-C). These results clearly indicated that p38 MAP kinase pathway was involved in the upstream regulation of PCNA, Cyclin D1, and Bcl-2 expression in response to 2α-hydroxyursolic acid in MDA-MB-231 human breast cancer cells.

A


KDa
PCNA
β-actin
36
42
Relative expression of PCNA
120
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80
60
40
20
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a
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2α-Hydroxyursolic acid (20 μM) - - + + + +
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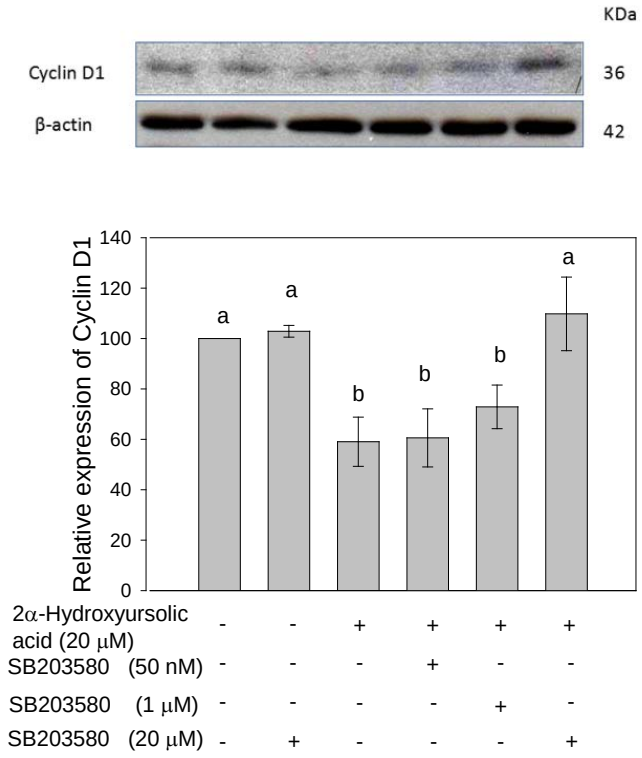
SB203580 (20 μM) - + - - - +

B


KDa
Cyclin D1
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Relative expression of Cyclin D1
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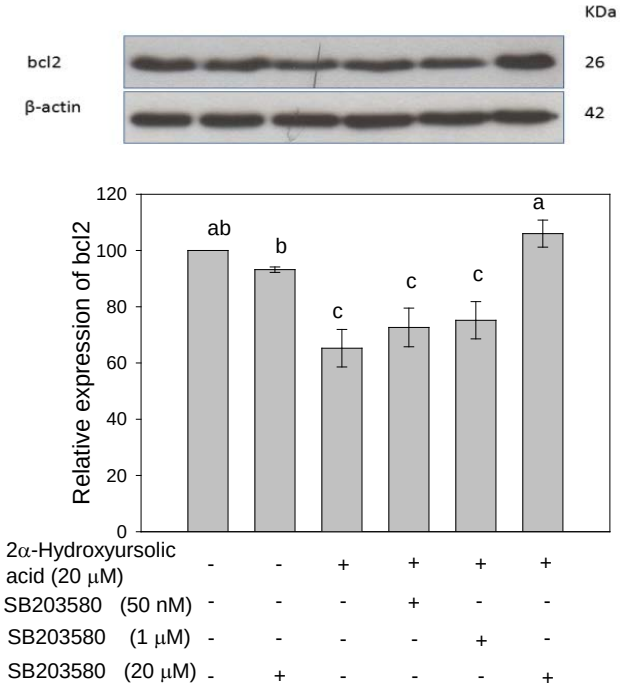

Figure 2.6. Effects of p38 inhibitor on 2α-hydroxyursolic acid-induced protein expression of PCNA (A), Cyclin D1 (B), and bcl2 (C) in MDA-MB-231 human breast cancer cells. MDA-MB-231 human breast cancer cells were pretreated with 50 nM, 1 μM or 20 μM p38 MAP kinase specific inhibitor, SB203580, for 1.5 h, and followed by treatment with or without 25 μM of 2α-hydroxyursolic acid for additional 24h. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

2.4 Discussion

It has been suggested that the changes in dietary behavior, such as increasing consumption of fruits, vegetables, and whole grains, are practical approaches to minimize the risk of developing chronic diseases [7, 16]. Dietary phytochemicals might account for the inhibitory effects of fruits, vegetables, whole grains, and other plant foods in the stages of promotion and progression of carcinogenesis [15]. Apple is a commonly eaten fruit in the United States. Apple phytochemical extracts have exhibited potent antioxidant activities and anti-proliferative activities against human cancer cells and prevent mammary cancers formation in a rat model *in vivo* [42, 49]. 2α-Hydroxyursolic acid, one of major active phytochemicals isolated from apple peels, has attracted great attention for its potential as a chemopreventive and chemotherapeutic agent in various types of cancer cells [23]. However, the mechanisms of action of 2α-hydroxyursolic acid's anticancer activity were not fully understood. Here we report that 2α-hydroxyursolic acid significantly inhibited MDA-MB-231 human breast cancer cells proliferation in a dose-dependent manner and no cytotoxicity was observed at the concentration below 30 μM. 2α-Hydroxyursolic acid significantly down-regulated expression of TRAF2, PCNA, Cyclin D1 and CDK-4 and up-regulated expression of p-ASK1, p-p38, p-p53 and p-21 in dose-dependent manners when compared to the control. 2α-Hydroxyursolic acid exposure induced apoptosis in MDA-MB-231 human breast cancer cells by significantly increasing the Bax/Bcl-2 ratio and inducing the cleaved caspase-3.

2.4.1 2α-Hydroxyursolic acid inhibits proliferation of MDA-MB-231 human breast cancer cells

Cell cycle control and apoptosis are the two major regulatory mechanisms of cell growth. Tumors are marked by a proliferation disorder and an apoptosis obstacle. In this study, 2α-hydroxyursolic acid significantly inhibited proliferation of MDA-MB-231 human breast cancer cells in a dose-dependent manner ($p<0.05$). At the doses of 15, 20, and 25 μM, 2α-hydroxyursolic acid showed no cytotoxicity (Figure 2.1). Previous studies showed that 2α-hydroxyursolic acid inhibited the proliferation of MCF-7 human breast cancer cells with an EC50 of 37.1 μM [41]. The EC_{50} of 2α-hydroxyursolic acid for inhibition of MDA-MB-231 human breast cancer cell proliferation was 19.82 μM, suggesting a specific anti-proliferative activity of 2α-hydroxyursolic acid towards MDA-MB-231 human breast cancer cells. Other studies also showed high anti-proliferative activity of 2α-hydroxyursolic acid towards HepG2 liver cancer cells and Caco-2 colon cancer cells [23], SKOV3, RMG-1 and ES-2 epithelial ovarian cancer cells [50], HL-60, U937, and Jurkat and THP-1 leukemia cell lines [40]. A network of signaling pathways is involved in regulating the inhibition of proliferation and the induction of apoptosis [51]. PCNA, proliferating cell nuclear antigen specifically expressed in proliferating cell nuclei, is often used as a proliferation marker. Cyclin D1, required for the progression of cells from G1 phase to S phase, is a component subunit of Cyclin-dependent kinases 4 (CDK4) and CDK6. Therefore, Cyclins and the Cyclin-dependent kinases play important role in controlling cell cycle [52]. Dysregulation of the cell cycle regulators is connected with

tumorigenesis. p21, as CDK inhibitor, implicated in the negative regulation of cell cycle progression from G1 to S phase [53]. In this study, 2α-hydroxyursolic acid has been demonstrated to up-regulate the expression of p21 and then down-regulate the downstream proteins expression of CDK4, Cyclin D1 and PCNA, and then inhibited cell proliferation of MDA-MB-231 human breast cancer cells (Figure 2.2).

2.4.2 2α-Hydroxyursolic acid induces apoptosis in MDA-MB-231 human breast cancer cells

Apoptosis, or programmed cell death, is a meaningful process in keeping balance between cell death and cell renewal in mature animals [54]. Apoptosis could be triggered through the activation of either intrinsic (mitochondrial) pathway or extrinsic (receptor-activated) pathway [55]. The intrinsic pathway is initiated by signals within the cell, while the extrinsic pathway is induced by the signals outside the cell. In the intrinsic pathway, the outer mitochondrial membrane responds to signal, turns to be depolarized and releases cytochrome c. In the cytoplasm, cytochrome c molecules form apoptosome by associating with the Apaf-1 protein. The complex converts a potential cytoplasmic protease procaspase 9 to its active form-cleaved caspase 9, which thereby cleaves procaspase 3 [56-58]. The sequence that one protease cleaved next one finally leads to apoptosis eventually. Bcl-2, an anti-apoptotic protein, works to avoid the leakage of cytochorme c by keeping the mitochondrial channel closed [59]. Bax, a pro-apoptotic member, works to accelerates programed cell death [60]. Researchers have reported that 2α-hydroxyursolic acid could trigger apoptosis by

increasing the Bax/Bcl-2 ratio in osteosarcoma MG-63 cells [61] and human cervix adenocarcinoma HeLa cells [62]. However, whether 2α-hydroxyursolic acid could induce apoptosis and mediated by the mitochondrial pathway still remains unknown. Our result showed that 2α-hydroxyursolic acid dramatically increased the Bax/Bcl-2 ratio (Figure 2.5C) and induced an activation of the cleaved caspase-3 (Figure 2.5D). The results of TUNEL assay also showed a significant increase of apoptotic cells after the treatment of 2α-hydroxyursolic acid, which indicated that 2α-hydroxyursolic acid induces apoptosis in MDA-MB-231 human breast cancer cells through mitochondrial death pathway.

As shown in Figure 2.3, the proteins involved in upstream signaling transduction pathway have been measured. Treatment of MDA-MB-231 human breast cancer cells with 2α-hydroxyursolic acid resulted in decreasing the protein expression of TRAF2 and increasing the protein expression of p-ASK1 (Figure 2.3C, 2.3D). The up-regulation of phosphorylated p38 and phosphorylated p53 has also been observed (Figure 2.3A, 2.3B). p38 MAPK signaling cascade plays a key role in the regulation of inflammation as well as in cell proliferation, differentiation, apoptosis and invasion [63]. Thus, a potential explanation might be the down-regulated TRAF2 and up-regulated p-ASK1 triggers downstream kinase p38 pathway, which leads to phosphorylation of p53, thereby inducing the anti-proliferation and apoptosis in MDA-MB-231 human breast cancer cells (Figure 2.7).

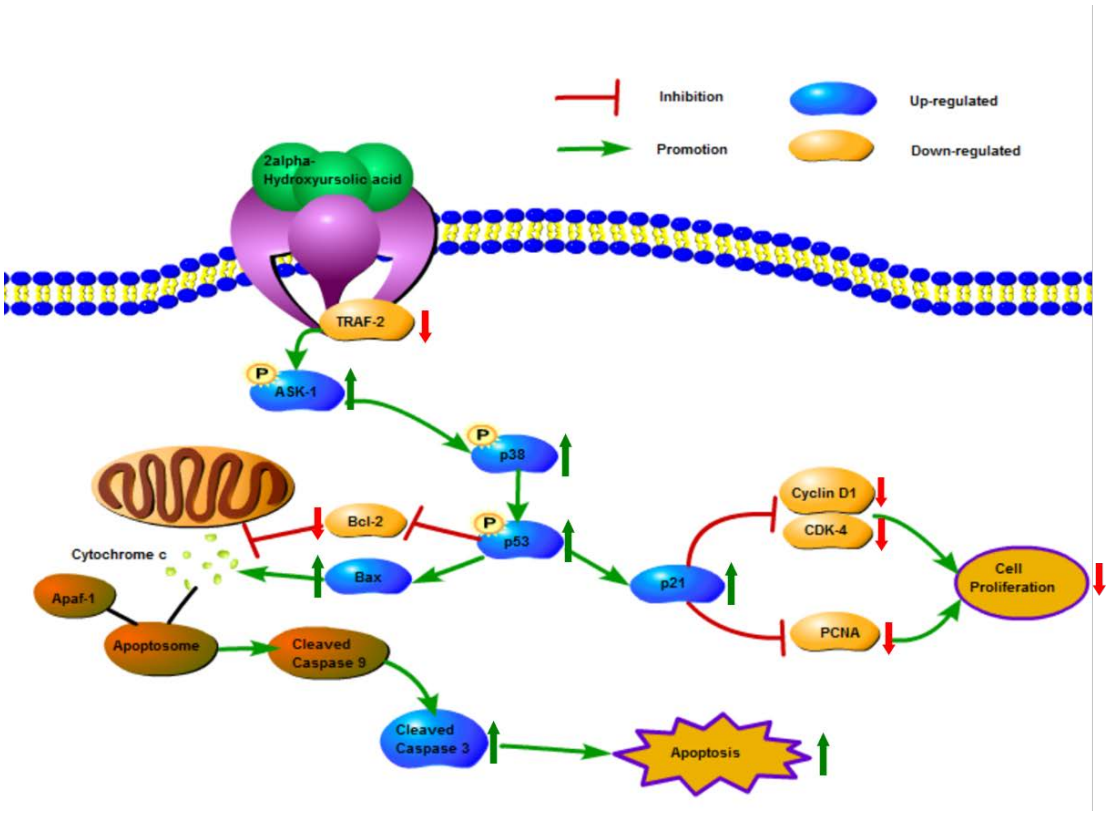

Figure 2.7. Potential mechanisms of action of 2α-hydroxyursolic acid in regulating cell proliferation and apoptosis in MDA-MB-231 human breast cancer cells through the p38 MAPK signal transduction pathway.

2.4.3 2α-Hydroxyursolic acid impacts the proliferation and apoptosis through p38 MAP kinase pathway

Previous studies have shown that the activation of ASK1 elevated expression of p38 MAPK in cell signaling pathways [64, 65]. To confirm whether 2α-hydroxyursolic acid inhibited cell proliferation and induced apoptosis through p38 MAPK pathway,

we investigated the effect of p38 MAP kinase specific inhibitor SB203580 on the expression of cell proliferation-related PCNA, cell cycle-related Cyclin D1 and apoptosis-related Bcl-2 expression. The data clearly showed that SB203580 abolished the inhibition of PCNA (Figure 2.6A), inhibition of Cyclin D1 (Figure 2.6B) and inhibition of apoptosis-related protein Bcl-2 (Figure 2.6C) in 2α-hydroxyursolic acid treated cells. Therefore, it is concluded that 2α-hydroxyursolic acid targeted p38 MAPK pathway to inhibit cell proliferation and induce apoptosis in MDA-MB-231 human breast cancer cells. It has also been reported that JNK, another MAPK, may also be triggered by ASK1 activation and involved in induction of cell apoptosis [66]. Signaling networks are highly interconnected with cross-talks among several pathways. 2α-Hydroxyursolic acid may target p38, other key proteins including JNK. Further research to determine the interactions of key proteins in cell signaling pathways is needed.

To the best of our knowledge, it might be the first time to report the treatment of MDA-MB-231 human breast cancer cells with 2α-hydroxyursolic acid results in significant inhibition of cell proliferation and the induction of apoptosis through p38 MAPK signaling pathway. 2α-Hydroxyursolic acid suppressed TRAF2 expression, activated expression of ASK1, and then up-regulated expression of phosphorylated p38 and p53. In the anti-proliferation branch, increased phosphorylated p53 triggered the expression of p21, and then lead to down-regulated expression of CDK4, Cyclin D1 and PCNA. In the apoptosis pathway, up-regulated p-p53 increased the Bax/Bcl-2 ratio and induced an activation of the cleaved caspase-3, and then leaded to apoptosis. Our findings suggested that consumption of fruits and vegetables with bioactive 2α-

hydroxyursolic acid might be beneficial to prevent or coordinate with treatment of triple-negative breast cancer. In further studies, more effort needs to be devoted to determine the receptors on the MDA-MB-231 human breast cancer cells for 2α-hydroxyursolic acid. Further *in vivo* studies are necessary to verify the efficacy and appropriate doses of 2α-hydroxyursolic acid for alleviating triple-negative breast cancer in clinical trials.

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CHAPTER 3

2α-HYDROXYURSOLIC ACID INHIBITED CELL METASTASIS THROUGH BLOCKING HGF/Met-PI3K/Akt-NF-κB SIGNAL TRANSDUCTION PATHWAY

Abstract

The effect of 2α-hydroxyursolic acid in inhibiting cell metastasis in MDA-MB-231 human breast cancer cells and potential mechanisms were investigated. Anti-migration, anti-invasion and anti-colony formation of 2α-hydroxyursolic acid in MDA-MB-231 cells was evaluated by scratch assay, trans-well assay and soft agar assay. Effect of 2α-hydroxyursolic acid on MMP-2 enzyme activity was determined by zymography assay. All the key proteins involved in regulating cell signal transduction pathway were determined by Western Blot analysis. 2α-Hydroxyursolic acid significantly inhibited HGF-induced MDA-MB-231 cell migration, invasion and colony formation at the concentrations without cytotoxicity. Western blot analysis indicated that 2α-hydroxyursolic acid significantly inhibited HGF-induced phosphorylation of Met and Akt, nuclear protein levels of NF-κB and VEGF expression. Furthermore, the activity of MMP-2, critical enzymes for cancer cell migration and invasion, was dramatically inhibited in a dose-dependent manner. These results suggested that 2α-hydroxyursolic acid exhibited anti-cancer activity through the inhibition of cell metastasis by blocking HGF/Met-PI3K/Akt-NF-κB signal transduction pathway.

Key Words: 2α-hydroxyursolic acid, metastasis, breast cancer, migration, invasion, phytochemicals

3.1 Introduction

A total of 595,690 cancer-related death are projected to occur in the United States in 2016, making cancer the second leading cause of death in the United States [1]. Normally, primary tumors only account for 10% of patient deaths from cancer, whereas metastasis is responsible for the rest 90% [2]. Breast cancer accounts for nearly one in three cancers among US women, and the leading cause of cancer death in 20-59 years old women [3-5]. Approximately 50% of women with breast cancer will develop metastatic disease, while the most frequent target organs are lung, liver and bone [6, 7].

Cancer metastasis is characterized by the following events: epithelial-mesenchymal transition (EMT), migration, invasion, induction of angiogenesis, and growth at metastatic sites [8]. As the tumor becomes bigger, cancer cells start competing for oxygen and nutrients to survive and some of them start suffering hypoxia. In this case, angiogenic factors, such as vascular endothelial growth factor (VEGF), will be released. VEGF could stimulate endothelial cells nearby to multiply and to construct new capillaries, which provide the nutrients and oxygen and also provide the cancer cells a path to find a new site that could be distant from primary tumor [9, 10]. At the same time, carcinoma cells go through an EMT, which enables them to become motile and invasive. Secretion of matrix metalloproteases (MMPs), such as MMP-2 and MMP-9, is an essential step of cancer invasion. MMPs are

proteolytic enzymes, which could catalyze the destruction of extracellular matrix (ECM) for cells to move [9, 11].

MDA-MB-231 breast cancer cell, belongs to triple negative breast cancer cell lines (negative for the expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2), is widely used as a model for metastatic cell migration [12]. Unlike other subtypes, triple negative breast cancer has limited number of receptors, making molecular targeted drugs therapies challenging [6, 13]. There is an overexpression of transmembrane protein, Met (or c-Met), in human breast cancers cells, including MDA-MB-231 breast cancer cell line [12, 14]. The ligand of the Met receptor is hepatocyte growth factor (HGF). Upon stimulation by HGF, Met becomes phosphorylated and initiates many biological events including EMT, migration, motility, invasion and angiogenesis [15, 16]. These observations suggest the potential of targeting HGF/Met to prevent tumor metastasis. As one of the downstream signaling components, phosphatidylinositol-3-kinase (PI3K)/Akt has been reported to be responsible for cell growth, proliferation, apoptosis, motility, epithelial EMT, angiogenesis and metastasis [2, 17]. Once activated, Akt can phosphorylate IκB kinase (IKK), which indirectly results in the translocation of nuclear factor kappa B (NF-κB) to the nucleus and the activation of gene transcription, such as MMPs and VEGF [18-20]. Therefore, targeting the PI3K/Akt pathway might be a potential strategy for inhibiting cancer metastasis.

Increasing consumption of fruits, vegetables, and whole grains has been suggested to reduce the incidence of cancers and other chronic diseases [21, 22]. Dietary phytochemicals might play a significant role in the inhibitory effects of fruits,

vegetables, whole grains, and other plant foods in the stages of promotion and progression of carcinogenesis [23]. Triterpenoids, which represent the largest group of phytochemicals, display a wide range of important medicinal activities, including anti-metastasis towards cancer [24-26]. 2α-Hydroxyursolic acid, one of major triterpenoids, has been isolated from apple peels reported previously [27]. 2α-Hydroxyursolic acid also has been discovered in other fruits and vegetables [26, 28, 29] with a variety of pharmacological effects including anti-cancer effect against several human cancer cell lines [27, 30-33]. Our previous study has shown that 2α-hydroxyursolic acid inhibited cell proliferation and induced apoptosis in MDA-MB-231 human breast cancer cells [34]. However, the ability of 2α-hydroxyursolic acid to inhibit metastasis in human breast cancer has not been reported as well as the underlying mechanism. The objective of this study was to investigate the effect of 2α-hydroxyursolic acid in inhibiting cell migration, invasion and angiogenesis and the molecular mechanism in a highly metastatic breast cancer cell line, MDA-MB-231 human breast cancer cells.

3.2 Materials and Methods

3.2.1 Chemicals

2α-Hydroxyursolic acid was isolated from apple peels by our lab as described previously [27]. Dimethyl sulfoxide (DMSO) was purchased from VWR (Radnor, PA, USA). Phosphate-buffered saline (PBS) and α-Minimum Essential Medium (α-MEM) were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Fetal

bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). Polyoxyethylene (tween 20) sorbitan monolaurate was obtained from Calbiochem (Billerica, MA, USA). Hepes, methyl green, protease inhibitors (aprotinin; leupeptin; pepstain; sodium orthovanadate), hepatocyte growth factor (HGF), gelatin, IGEPAL (CA-630) and agarose were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

3.2.2 Antibodies

Primary antibody against p-Met, Met, p-Akt, Akt, NF-κB and VEGF was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against β-actin, anti-mouse IgG and anti-rabbit IgG were obtained from Sigma-Aldrich Inc. (St. Louis, MO).

3.2.3 Cell culture

MDA-MB-231 human breast cancer cells purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained at 37 °C in a humidified 5% CO_2 atmosphere in α-MEM containing 10 mM Hepes, 1% antibiotic-antimycotic and 10% heat-inactivated fetal bovine serum as described previously [33, 35].

3.2.4 Wound-healing assay

Before plating the cells, a line was drawn at the underside of the wells with a needle, to serve as fiducial marks demarcating the wound areas to be analyzed. MDA-MB-231

cells in 1.5 mL fresh medium were plated in 6-well plate at a density of 5.0×10^5 cells/ well and were incubated at 37 °C in 5% CO_2 for 24 h. Then the growth medium was replaced with 1.5 mL serum free medium (SFM) to starve the cells for another 24 h. After the SFM was removed, the confluent monolayers were wounded using a 200 µL disposable plastic pipette tip (approximately 1 mm in size) and washed with PBS twice. Next, the cells were treated with either SFM or HGF (40 ng/mL) in the presence of 2α-hydroxyursolic acid at doses of 0, 15, and 20 µM. The wounds were observed using bright field microscopy and pictures were taken at three randomly selected distinct view fields flanking the intersections of the wound and the marker lines three times at 0, 12, and 24 h. Data were analyzed using Sigmaplot software version 11.0 (Systat Software, Inc. Chicago, IL). The migration rate was measured by the percentage of wound area at 12 and 24 h versus the origin area at 0 h. The area is analyzed by Adobe Photoshop CS4 (Adobe systems Inc. California, USA). Results will be conducted in triplicate.

3.2.5 Cell invasion assay

The CHEMICON Cell Invasion Assay Kit, purchased from EMD Millipore (Merck KGaA, Darmstadt, Germany), was used to detect cell invasion. 300 µL of warm serum free media was added to the interior of the inserts to rehydrate the extracellular matrix (ECM) layer for 1 hour at room temperature. Then 500 µL of SFM containing 40 ng/mL HGF was added to the lower chamber and 300 µL SFM containing MDA-MB-231 breast cancer cells (5.0×10^5 cells/mL) and different concentrations of 2α-

hydroxyursolic acid (0, 15, 20 and 25 μM) was added to each inserts. After 24 hours incubation at 37°C in 5% CO_2, a cotton-tipped swab was used to gently remove non-invading cells as well as the ECMatrix gel from the interior of the inserts. The invasive cells could be stained on lower surface of the membrane by dipping inserts in the staining solution for 30 minutes. Dip inserts in a beaker of water several times to rinse and allow the inserts to dry. Quantitate the cells by dissolving stained cells in 10% acetic acid (100-200 μL/well) and transfer a consistent amount of the dye/solute mixture to a 96-well plate for colorimetric reading of OD at 560 nm. All measurements were conducted in three replications.

3.2.6 Soft agar colony formation assay

MDA-MB-231 breast cancer cells (6×10^3 cells/well) were seeded in the upper layer containing 0.3% agar in complete medium with different concentrations of 2α-hydroxyursolic acid (0, 15, 20 and 25 μM). The solid agar base (bottom layer) contained 0.6% agar in 2 mL complete medium per well. After layers were solidified, the plates were incubated with 37 °C and 5% CO_2 for 5 weeks. The cultures were fed once a week with 0.3% agarose medium containing 40 ng/mL HGF and different concentrations of 2α-hydroxyursolic acid. Colony grade was measured in 9 random fields per well with an 40× magnification inverted light microscope at 1, 3 and 5 week. The colony grade was defined as the sum of the n (n=colony diameter (μm)/ 50 μm). The colony grade ratio equals the percentage of colony grade of different doses of 2α-hydroxyursolic acid against the colony grade of control (0 dose of 2α-

hydroxyursolic acid). After 5 weeks of growth, colonies with diameter at least 50 μm were counted each well under inversion microscopy.

3.2.7 Gelatin zymography assay

Gelatin zymography, a widely used technique in the study of metalloproteinase-2 (MMP-2), is described previously [5]. MDA-MB-231 cells in 1.5 mL fresh medium were plated in 6-well plate at a density of 1.0×10^6 cells/ well and were incubated at 37°C in 5% CO_2 for 24 h. Then the growth medium was replaced with 1.5 ml serum free medium (SFM) to starve the cells for another 24 h. The SFM were removed and the cells were treated with different concentrations of 2α-hydroxyursolic acid (0, 15, 20 and 25 μM) and 40 ng/mL HGF in SFM. The medium was harvest after 24 h and centrifuged for 3 min at 400 g. Collected medium was then mixed with 2% SDS loading buffer and subjected to 10% SDS-PAGE electrophoresis with 0.8 mg/mL gelatin. After electrophorese using a constant voltage power supply at 150 V for roughly 1 h, the gel was gently washed using fresh enzyme renaturing buffer containing 2.5% (v/v) Triton X-100 to 4 times, for a total washing time of 1 h to remove SDS. Then the gel was transferred to developing buffer (enzyme renaturing buffer without Triton X-100) agitated on shaker for 1 hour and followed by incubating in developing buffer for 20 h at 37 ℃ to allow proteolysis of the gelatin substrate. Clear bands of gelatinolytic activity were visualized after staining the gel with 0.125% (w/v) Coomassie brilliant blue in 20% (v/v) acetic acid and 50% (v/v) methanol, followed by destaining in 30% (v/v) methanol and 1% (v/v) formic acid. Bands were

then photographed by digital camera and quantified by ImageJ2x software (Wayne Rasband, National Institutes of Health, Maryland, USA). The MMP-2 activity was determined as percentage compared to control. All measurements were conducted in three replications.

3.2.8 Preparation of whole-cell lysates and nuclear fraction

Whole-cell lysates and nuclear fractions of cells were prepared by using the method as reported previously in our laboratory [36]. Briefly, MDA-MB-231 human breast cancer cells were seeded at a density of 5.0×10^5 cells/ well in 6-well plates for 8 hours and then the cells were incubated overnight without serum. Next, various concentrations of 2α-hydroxyursolic acid were added into the wells. After 2 h of incubation at 37 °C in 5% CO_2, the cells were stimulated by 40 ng/mL HGF for 30 min. Then the cells were washed twice with ice-cold PBS and scraped off from the wells.

For whole-cell lysates, harvested cells were then lysed using lysis buffer (50 mM Tris, pH 7.4; 1% Igepal; 150 mM sodium chloride; and 1 mM EDTA) with different protease inhibitors (1 g/mL aprotinin; 1 g/mL leupeptin; 1 g/mL pepstain; and 1 mM sodium orthovanadate). Cell lysates were vortexed briefly every 5 min for half an hour to facilitate protein extraction. Lysates were then centrifuged at 12,000 g for 5 min at 4 °C. For nuclear fraction, the harvested cells were added buffer A (10 mM HEPES pH 7.8, 10 mM KCl, 0.1 mM EDTA) with 1mM DTT, 1 μg/mL aprotinin, 1 μg/ml leupeptin, 1 μg/mL pepstatin and 0.2 mM sodium orthovanadate. Cells were

kept on ice 15 min, added 1% IGEPAL (CA-630), and the cell suspension was mixed for 15 s. Then, the cell suspension was centrifuged at 2000 g for 3 min at 4 °C. The cell nucleus was resuspended in buffer B (50 mM HEPES-KOH pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) with 1mM DTT, 1 μg/mL aprotinin, 1 μg/ml leupeptin, 1 μg/mL pepstatin and 0.2 mM sodium orthovanadate and vortexed every 2-3 min. Nuclear lysates were centrifuged at 12,000 g for 30 min at 4 °C and the supernatant was collected as the nuclear extract.

3.2.9 Western blot assay

The western blot analysis was carried out as described previously [36, 37]. Protein concentrations of both whole-cell lysates and nuclear fractions were determined using a Sigma Diagnostics Micro Protein Determination Kit and a FilterMax F5 Multi-Mode Microplate Readers (Molecular Devices, Sunnyvale, CA). Equal amounts of protein from each cell lysate was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes. The membranes were blocked for 2 h with 5% non-fat dry milk in TBST (Tris-base buffer solution containing of 0.1% Tween 20) at room temperature and incubated with the desired primary antibody (all in 1:1,000 dilutions in 1% nonfat dry milk and TBST) overnight at 4˚C. After secondary antibody was applied, membrane-bound antibodies were visualized by the Enhanced Chemiluminescence kit (Cell Signaling Technology, Inc., Beverly, MA) according to the manufacturer's instruction. Bands were then scanned and quantified by ImageJ2x software (Wayne Rasband, National Institutes of

Health, Maryland, USA). The expression of human α-tubulin or β-actin was used as an internal standard control. All measurements were conducted in triplicate.

3.2.10 Statistical analysis

Data were analyzed using Sigmaplot software version 11.0 (Systat Software, Inc. Chicago, IL) and dose-effect analysis was performed using Calcusyn software version 2.0 (Biosoft, Cambridge, UK). Data were presented as mean ± SD for at least three independently performed experiments. Statistical analyses were carried out with Tukey's test and analysis of variance (ANOVA) by JMP software version 9.0.2 (SAS Institute Inc. North Carolina, USA). Differences with $p<0.05$ were considered to be statistically significant.

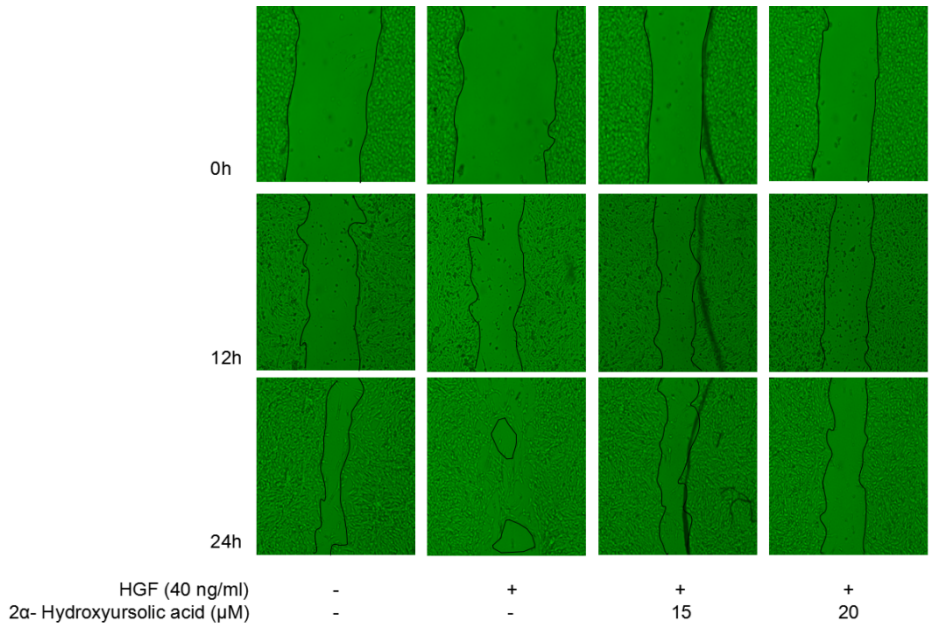
3.3 Results

3.3.1 2α-Hydroxyursolic acid inhibited HGF-mediated migration of MDA-MB-231 human breast cancer cells

Wound-healing assay was adopted to evaluate the effects of 2α-hydroxyursolic acid on the HGF-mediated migration activities of MDA-MB-231 human breast cancer cells. Cells were cultured in either SFM or HGF (40 ng/mL) in the presence of 2α-hydroxyursolic acid at doses of 0, 15, and 20 μM. Compared to the starved untreated MDA-MB-231 cells, the HGF-treated cells showed more significantly accelerated wound closure activity after treatment for 12 and 24 h (Figure 3.1A). However, treatment with 15 μM and 20 μM 2α-hydroxyursolic acid remarkably suppressed HGF

induced wound closure activity. As shown in Figure 3.1B, the wound width is 77.40 ± 3.34% after treated with 20 μM 2α-hydroxyursolic acid for 24 h, compared with only 18.48 ± 5.77% wound width in HGF control group for 24 h. The results demonstrated that the MDA-MB-231 cell motility is significantly suppressed in a dose-dependent manner compared with the HGF-induced control group (Figure 3.1, $p<0.05$).

A



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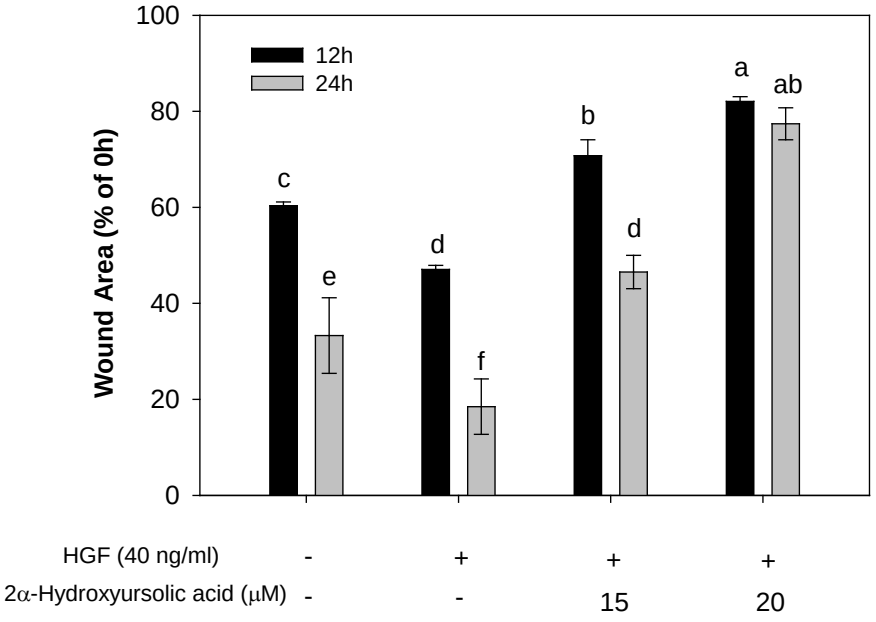


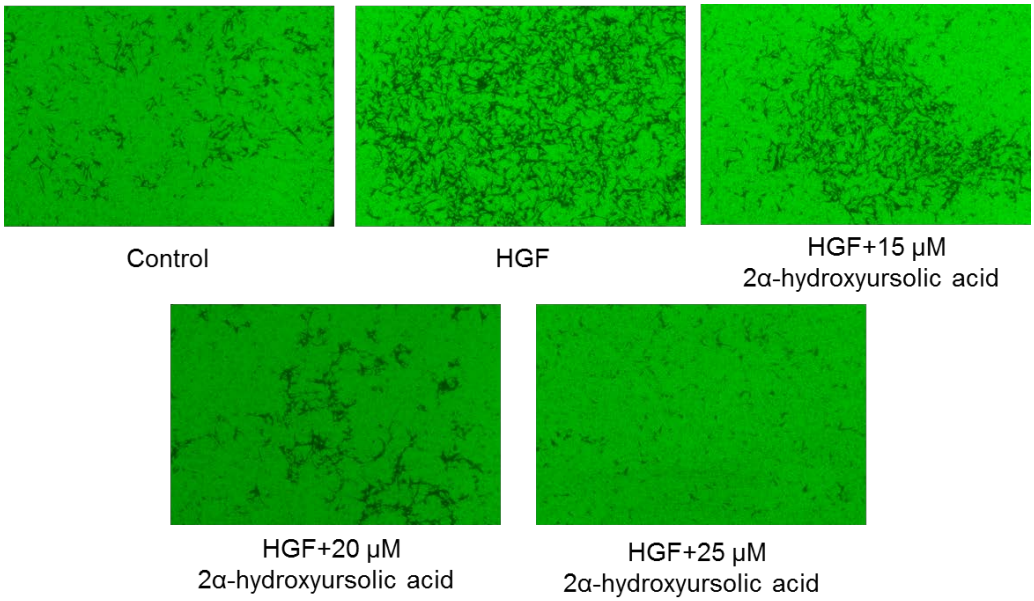
Figure 3.1. Effect of 2α-hydroxyursolic acid on the cell motility in MDA-MB-231 human breast cancer cells. In the wound-healing assay, the serum-starved monolayers

cells were wounded using a disposable plastic pipette tip and the cells were treated with either SFM or HGF (40 ng/mL) in the presence of 2α-hydroxyursolic acid at doses of 0, 15, and 20 μM. (A) Width of wounds was observed using bright field microscopy and the representative photographs showed the same area three times per well at 0, 12, and 24 h. (B) The migration rate was measured by the percentage of wound area at 12 and 24 h versus the origin area at 0 h. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

3.3.2 α-Hydroxyursolic acid inhibited HGF-mediated invasion of MDA-MB-231 human breast cancer cells

The effect of 2α-hydroxyursolic acid on cell invasion was detected using the Transwell chamber assay. HGF, as expected, significantly increased the invasive cancer cells (Figure 3.2A). After treating the cells with HGF, the number of cells that had passed through the transwell chamber polycarbonate membrane were increased by 152.14% (Figure 3.2B, $p<0.05$). The results also showed that 2α-hydroxyursolic acid significantly decreased the HGF-induced cell invasion in a dose-dependent manner (Figure 3.2, $p<0.05$).

A



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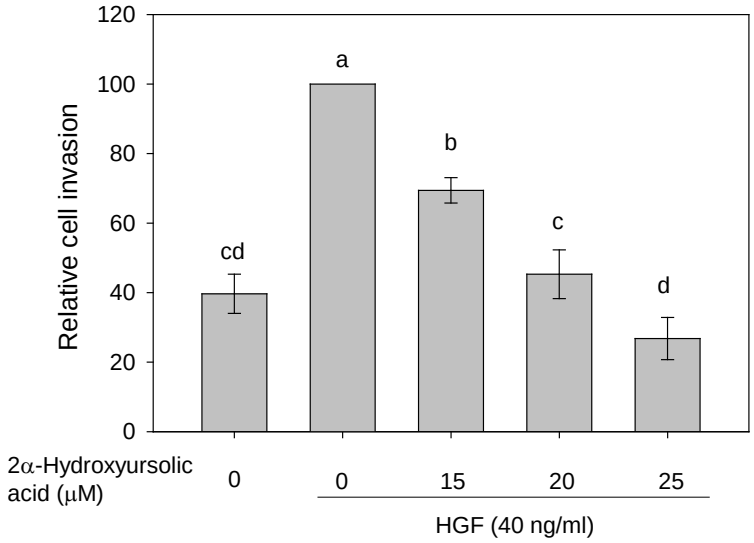


Figure 3.2. Effect of 2α-hydroxyursolic acid on the cell invasion in MDA-MB-231 human breast cancer cells. In the cell invasion assay, SFM containing MDA-MB-231 breast cancer cells and different concentrations of 2α-hydroxyursolic acid (0, 15, 20

and 25 μM) were added into the inserts. Either SFM or HGF (40 ng/mL) was applied to the lower chamber as a chemoattractive agent. The invading cells on the lower surface of the membrane filter were stained by stain solution after 24 h. (A) Representative photographs showed the stained invading cells under different treatment. (B) The invasion rate was measured by the colorimetric reading of OD at 560 nm after the staining and staining dissolving steps. Bars with no letters in common are significantly different ($p< 0.05$). Each value represents the mean ± SD of triplicates.

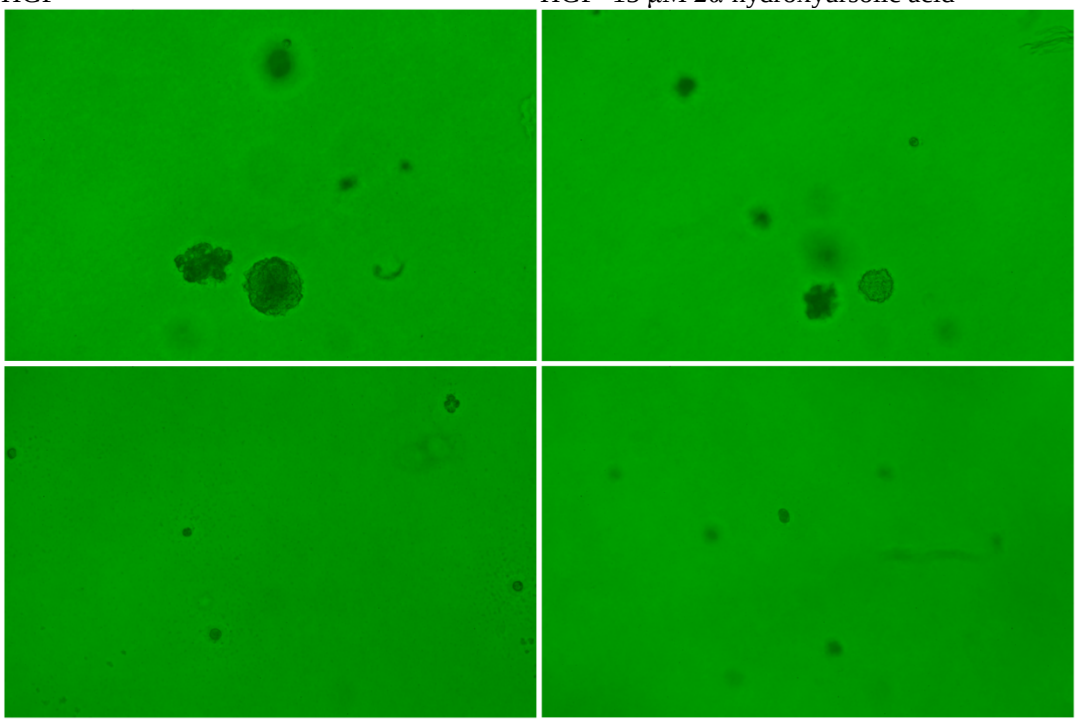
3.3.3 2α-Hydroxyursolic acid inhibited colony formation in MDA-MB-231 human breast cancer cells

Soft agar assay was used to identify the inhibitory effect of 2α-hydroxyursolic acid on anchorage-independent growth of MDA-MB-231 human breast cancer cells. As shown in Figure 3.3B, the colony grade, which take both colony size and quantity into account, increased during five weeks. However, 2α-hydroxyursolic acid inhibited this time-dependent colony formation. At the 5th week, the number of colonies (diameter at least 50 μm) is 246 for control. In comparison, the colony number was significantly decreased to 193, 133 and 70 after the treatment of 2α-hydroxyursolic acid at the concentrations of 15, 20, and 25 μM, respectively (Figure 3.3C). In the group receiving 25 μM of 2α-hydroxyursolic acid, the colony numbers were decreased by 71.54% when compared to the control ($p<0.05$).

A

HGF

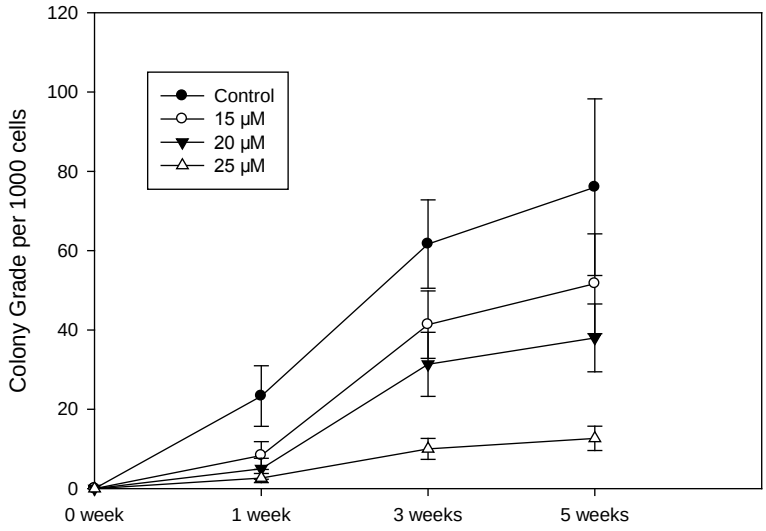
HGF+15 μM 2α-hydroxyursolic acid



HGF+20 μM 2α-hydroxyursolic acid

HGF+25 μM 2α-hydroxyursolic acid

B



C

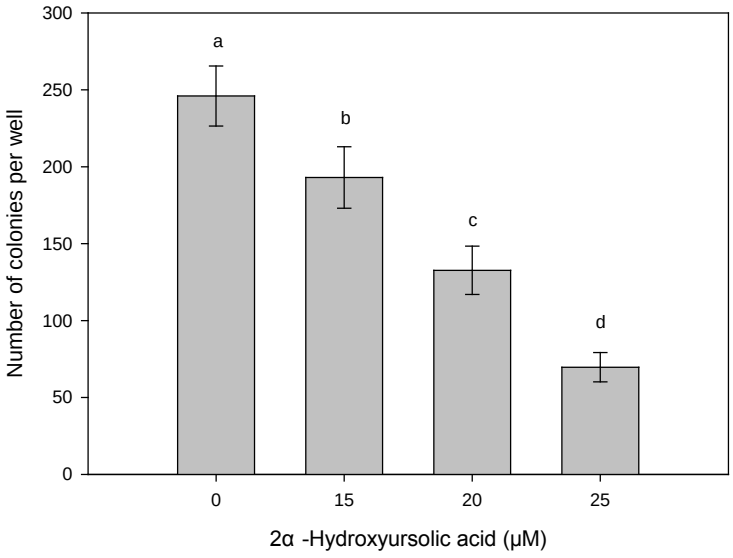


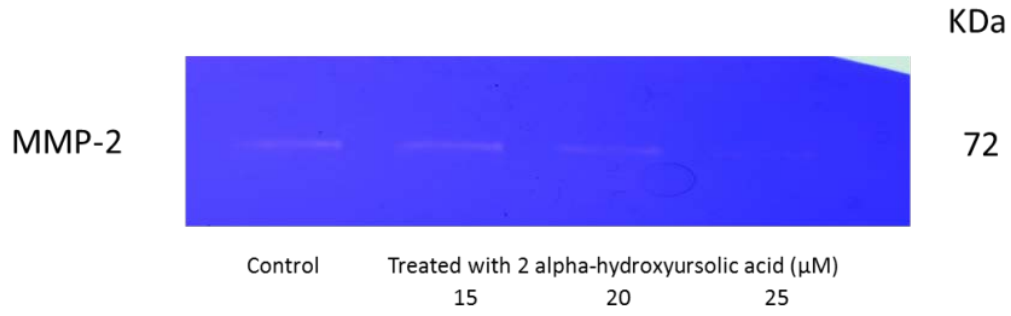
Figure 3.3. Effects of 2α-hydroxyursolic acid on the inhibition of colony formation in MDA-MB-231 human breast cancer cells. In soft agar assay, MDA-MB-231 human breast cancer cells were seeded in complete medium containing 0.3% agar with

different concentrations of 2α-hydroxyursolic acid (0, 15, 20 and 25 μM). (A) Representative photographs of colonies using bright field microscope were taken at 5th week after plating. (B) Colony grade was measured in 9 random fields per well with an 40× final magnification inverted light microscope at 1 week, 3 week and 5 week. The colony grade was defined as the sum of the n (n=colony diameter (μm)/ 50 μm). The values were quantified as colony grade per 1000 cells in triplicate. (C) After 5 weeks of growth, colonies with diameter at least 50 μm were counted each well under inversion microscopy. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

3.3.4 2α-Hydroxyursolic acid inhibited HGF-mediated MMP-2 activity in MDA-MB-231 human breast cancer cells

To further investigate the mechanisms of invasion inhibition mediated by 2α-hydroxyursolic acid, gelatin zymography was performed to determine MMP-2 activity in MDA-MB-231 human breast cancer cells. As shown in Figure 3.4A, 2α-hydroxyursolic acid significantly inhibited HGF-induced MMP-2 gelatinolytic activity. The MMP-2 activity was significantly reduced by 50.24 and 85.36% at the doses of 20 and 25 μM when compared to the control (Figure 3.4B, $p<0.05$). However, since an extremely low level of MMP-9 induced by HGF in MDA-MB-231 human breast cancer cells (data not shown), the effect of 2α-hydroxyursolic acid on MMP-9 activity was inconclusive.

A



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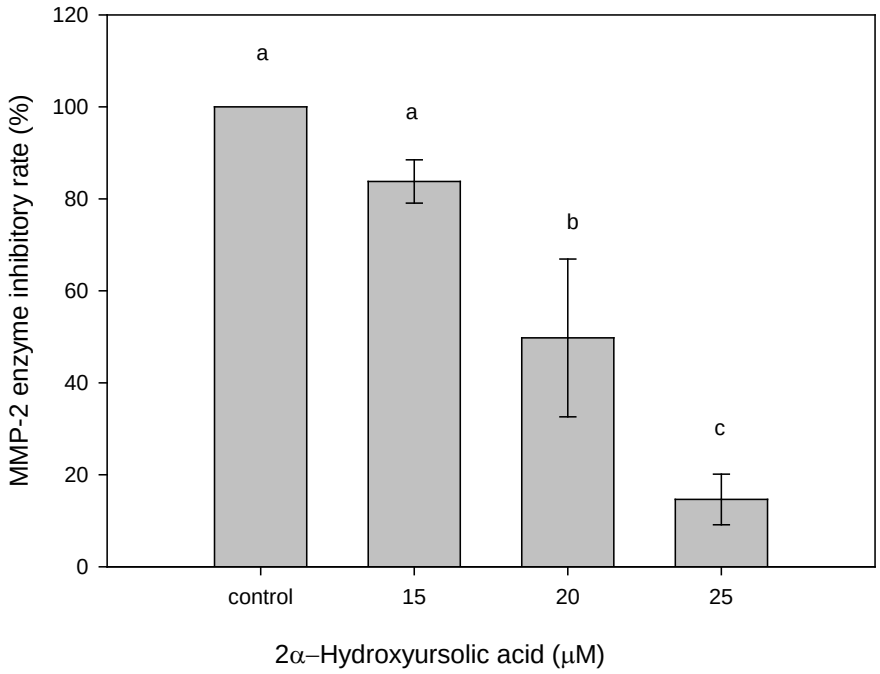

Figure 3.4. Effects of 2α-hydroxyursolic acid on the proteolytic activities of MMP-2 in MDA-MB-231 human breast cancer cells. The serum-starved cells were exposed to 0, 15, 20 and 25 μM 2α-hydroxyursolic acid and 40 ng/mL HGF in SFM. (A) After 24 h, the medium were collected, and electrophoresed on gelatin gels and stained. Bands were then photographed by digital camera. (B) Bands were quantified by ImageJ2x

software and the MMP-2 activity was determined as the percentage compared to control. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

3.3.5 2α-Hydroxyursolic acid inhibited HGF-induced nuclear NF-κB in MDA-MB-231 human breast cancer cells

NF-κB is a transcription factor. Once released, NF-κB moves into the nucleus from the cytoplasm and binds to the promoter region of target genes to activate gene expressions, including MMP-2 [5, 38]. In order to clarify the involvement of NF-κB protein in the mechanism of 2α-hydroxyursolic acid's anti-metastasis effect, the expression of NF-κB in the nuclear extracts were analyzed by Western blot assay. As shown in Figure 3.5A, 2α-hydroxyursolic acid remarkably down-regulated the nuclear NF-κB. When compared to HGF treated control, protein level of the nuclear NF-κB was decreased to 50.14 and 23.94% in MDA-MB-231 human breast cancer cells treated with 20 and 25 μM 2α-hydroxyursolic acid when compared to the control ($p<0.05$).

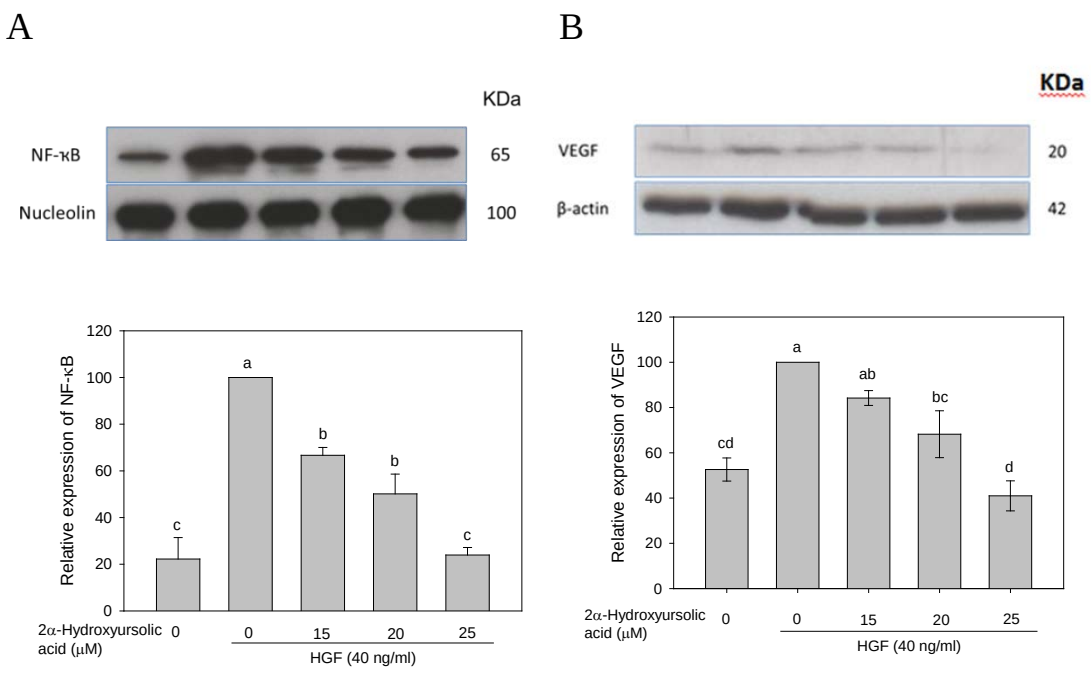

Figure 3.5. Effects of 2α-hydroxyursolic acid on expression of nuclear NF-κB (A) and VEGF (B) in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

3.3.6 2α-Hydroxyursolic acid inhibited HGF-induced VEGF expression in MDA-MB-231 human breast cancer cells

To investigate the potential anti-angiogenesis activity of 2α-hydroxyursolic acid towards MDA-MB-231 human breast cancer cells, the expression of angiogenic factor, VEGF, was measured. Data from Western blot analysis showed that 2α-hydroxyursolic acid significantly decreased the protein levels of VEGF in MDA-MB-231 human breast cancer cells in a dose-dependent manner (Figure 3.5B, $p<0.05$). In the group receiving 25 μM of 2α-hydroxyursolic acid, the HGF-induced VEGF expression was

inhibited by 59.03% when compared to the control ($p<0.05$).

3.3.7 2α-Hydroxyursolic acid inhibited HGF-induced Met phosphorylation in MDA-MB-231 human breast cancer cells

The HGF/Met signaling pathway is up-regulated in numerous types of cancer, including breast cancer. In order to determine the effect of 2α-hydroxyursolic acid on Met phosphorylation, western blot analysis was performed. The result showed that HGF induced the phosphorylation of Met, whereas 2α-hydroxyursolic acid significantly inhibited HGF-induced Met phosphorylation in a dose-dependent mannaer (Figure 3.6, $p<0.05$). In the group receiving 25 μM of 2α-hydroxyursolic acid, the phosphorylation of Met was inhibited by 56.73% when compared to the control ($p<0.05$). The total protein levels of Met remained unchanged with these treatments.

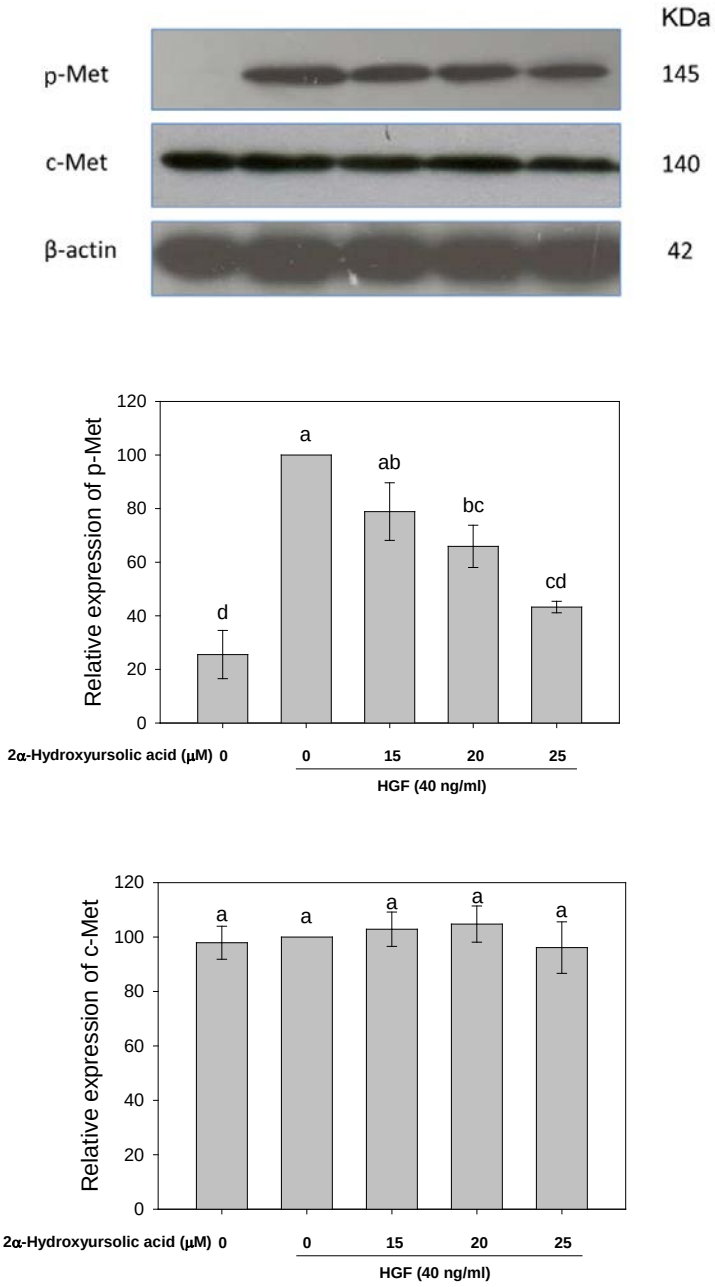

Figure 3.6. Effects of 2α-hydroxyursolic acid on expression of p-Met and c-Met in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

3.3.8 2α-Hydroxyursolic acid inhibited HGF-induced Akt phosphorylation in MDA-MB-231 human breast cancer cells

Akt is a multifunctional regulator of cell survival, growth and invasion. In order to further elaborate the molecular mechanism of how 2α-hydroxyursolic acid inhibits HGF-mediated invasive growth in MDA-MB-231 cells, we measured the effect of 2α-hydroxyursolic acid on Akt phosphorylation. Western blotting analysis of cells treated with various concentrations of 2α-hydroxyursolic acid indicated that the HGF-induced phosphorylation of Akt was down-regulated in a dose-dependent manner (Figure 3.7). In the group receiving 25 μM of 2α-hydroxyursolic acid, the phosphorylation of Akt was inhibited by 69.06% when compared to the control ($p<0.05$). The total protein levels of Akt remained unchanged with these treatments.

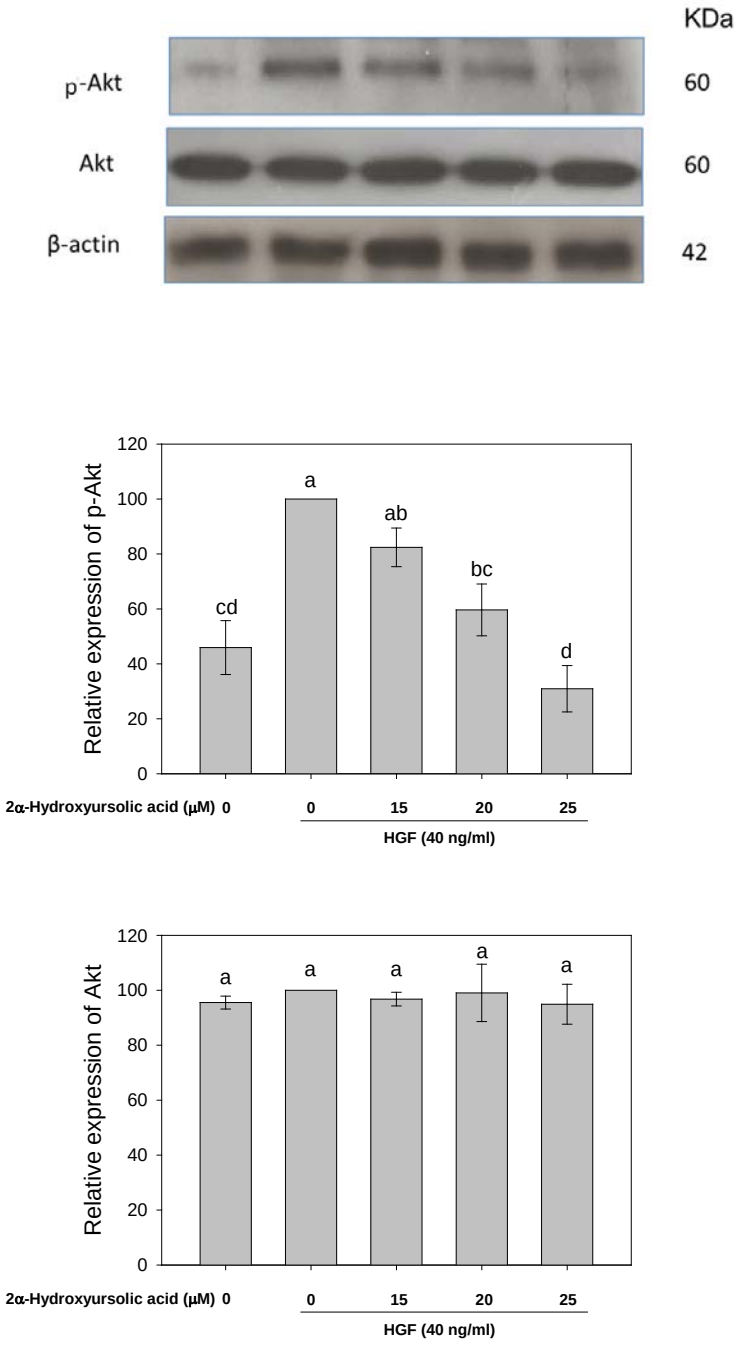

Figure 3.7. Effects of 2α-hydroxyursolic acid on expression of p-Akt and Akt in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

3.4 Discussion

Cancer is a major public health problem not only in the United States, but also many other parts of the world. According to Dietary Guidelines for Americans [39], a healthy eating pattern includes a variety of vegetables from all of the subgroups, fruits (especially whole fruits) and grains (at least half of which are whole grains). Apples are an important part of the diet in human. Unpeeled apple showed even higher antioxidant activity and anti-proliferative activity compared to peeled apples [40], which means apple peels are rich in bioactive compounds. The main bioactive components in apple peels are believed to phenolic compounds and triterpenes [27, 41]. 2α-Hydroxyursolic acid, one of major active triterpenoids isolated from apple peels, has attracted much attention due to its anti-cancer activity. Previous studies have demonstrated that 2α-hydroxyursolic acid could inhibit cancer progression against several human cancer cell lines [27, 30-33, 42]. However, the effect of 2α-hydroxyursolic acid in inhibiting metastasis in MDA-MB-231 human breast cancer cells has not been reported. In this study, we report that 2α-hydroxyursolic acid significantly inhibited HGF-induced MDA-MB-231 human breast cancer cells migration, invasion and colony formation at the tested concentrations without cytotoxicity [34].

2α-Hydroxyursolic acid significantly down-regulated HGF-induced Met phosphorylation, Akt phosphorylation, nuclear NF-κB in dose-dependent manners when compared to the HGF-induced control group. In addition, 2α-hydroxyursolic acid decreased HGF-induced MMP-2 activity and VEGF expression in MDA-MB-231 human breast cancer cells.

3.4.1 2α-Hydroxyursolic acid inhibited the HGF-mediated migration, invasion and colony formation in MDA-MB-231 human breast cancer cells

In wound healing, epithelial cells need to go through EMT, which allows these cells to move into the wound site. There is striking resemblance between tumor progression and wound healing [43]. Thus, in order to identify whether 2α-hydroxyursolic acid influences tumor cell migration, we adopted wound-healing assay to study the cell migration. The starved untreated MDA-MB-231 human breast cancer cells exhibit only a limited wound closure activity, whereas HGF-treated cells migrated into the cell-free area were far more than that of untreated cells. When compared to the HGF-induced control group, treatment of the cells with 15 and 20 μM 2α-hydroxyursolic acid inhibited cell migration by 23.68 and 34.99% for 12 h, 28.05 and 58.92% for 24 h, respectively (Figure 3.1, $p<0.05$). Invasion though ECM is also an essential step in tumor metastasis [44]. In this study, we evaluated the invasion of tumor cells through a basement membrane model. ECMatrix, proteins derived from the Engelbreth Holm-Swarm (EHS) mouse tumor was used as basement membrane matrix [45] to measure the effect of 2α-hydroxyursolic acid in inhibiting cell invasion. The results also showed that 2α-hydroxyursolic acid significantly suppressed the HGF-induced cell invasion in a dose-dependent manner (Figure 3.2, $p<0.05$). Soft agar colony formation assay is designed to detect the ability of cells to grow unattached to a surface termed as anchorage-independent growth. Anchorage-independent growth is another hallmark of EMT [46], which is the most accurate in vitro assay for detecting malignant

transformation of cells. Thus, soft agar colony formation assay is employed to detect the effect of 2α-hydroxyursolic acid on cancer cell colony formation. The result showed that 2α-hydroxyursolic acid inhibited HGF-induced colony formation in a dose-dependent manner (Figure 3.3, $p<0.05$). Therefore, 2α-hydroxyursolic acid significantly decreased HGF-mediated cell migration, invasion and colony formation in a dose-dependent manner.

3.4.2 2α-Hydroxyursolic acid inhibited the HGF-induced MMP-2 activity and VEGF expression in MDA-MB-231 human breast cancer cells

Since ECM maintains tissue polarity and architecture, so the capacity for traversing several physical barriers like ECM is required for cancer cell metastasis [47]. MMPs, which secreted by recruited stromal cells and carcinoma cells, degrade specific components of ECM, including basement membranes, specifically collagen. MMP-2, known as one of the most extensively studied MMPs, degrades gelatin, elastin and collagen [48]. Elevated MMP-2 activity is associated with tumor metastasis in all kinds of cancers, including breast cancer [49, 50]. Zymography is an electrophoretic technique to visualize the size of the proteinases bands on the gel based on their hydrolysis of a gel-embedded protein substrate. Therefore, gelatin zymography assay is employed to measure the effect of 2α-hydroxyursolic acid on hydrolytic ability of MMP-2. As shown in Figure 3.4B, 2α-hydroxyursolic acid dramatically reduced MMP-2 activity at the doses of 20 and 25 μM by 50.24 and 85.36% ($p<0.05$). VEGF, an angiogenic factor, helps develop new blood vessels for cancer cells to travel

throughout the body [10]. Western blot analysis was adopted to measure the expression of VEGF. It has been shown that 2α-hydroxyursolic acid significantly decreased the protein levels of VEGF in MDA-MB-231 human breast cancer cells in a dose-dependent manner (Figure 3.5B, $p<0.05$).

3.4.3 2α-Hydroxyursolic acid inhibited the HGF/Met PI3K/Akt and NF-κB pathways in MDA-MB-231 human breast cancer cells

The higher levels of circulating HGF and its receptor Met in patients with mammary cancer are consistent with a lower survival and development of distant metastasis [51]. HGF/Met pathway has been elucidated to trigger EMT, migration, invasion and angiogenesis [52-54]. In addition, the activation of Akt by phosphorylated Met is necessary for many biological events of the metastatic pathway [2]. Phosphorylated Akt in turn triggers the translocation of NF-κB to activate of gene transcription, such as MMPs [18, 19]. Therefore, this study has focused on inhibiting HGF/Met, PI3K/Akt and NF-κB pathways as potential targets for treating cancer metastasis. The proteins involved in upstream signaling transduction pathway have been measured by western blot assay. Treatment of MDA-MB-231 human breast cancer cells with 2α-hydroxyursolic acid resulted in decreasing the phosphorylation of Met and Akt, while the total protein levels of c-Met and Akt remained unchanged with these treatments (Figure 3.6 and 3.7). Also 2α-hydroxyursolic acid inhibited the HGF-induced NF-κB activation via decreasing the translocation of NF-κB to nuclear in a dose-dependent manner (Figure 3.5A).

Not much research has been reported to investigate the effect of 2α-hydroxyursolic acid on tumor metastasis and angiogenesis. However, there is one study demonstrated that 2α-hydroxyursolic acid, orally administrated to mice before and after subcutaneous tumor implantation with LM85 cells suppressed lung metastasis [42]. To the best of our knowledge, this is the first time that 2α-hydroxyursolic acid significantly inhibited cell metastasis in MDA-MB-231 human breast cancer cells. 2α-Hydroxyursolic acid inhibited HGF-induced invasive growth of MDA-MB-231 breast cancer cells, including migration, invasion, colony formation and angiogenesis. The proposed mechanism might be the down-regulated Met phosphorylation blocked the phosphorylation of Akt, which leads to the inhibition of NF-κB translocation to nuclear, resulting in the reduction of MMP-2 activity and down-regulation of VEGF expression, thereby inducing the anti-metastasis in MDA-MB-231 human breast cancer cells (Figure 3.8). These results are important in understanding anti-cancer activity of fruits and vegetables and potential application of 2α-hydroxyursolic acid in the prevention of breast cancer metastasis. It has been reported that Ras/MAPK and the JAK/STAT pathway might also be downstream components of HGF/Met. The involvement of other alternative pathways in 2α-hydroxyursolic acid inhibited metastasis needs further investigation. Also, *in vivo* studies needs to be conducted in further research to provide more scientific evidence for this compound as a potential candidate for cancer treatment.

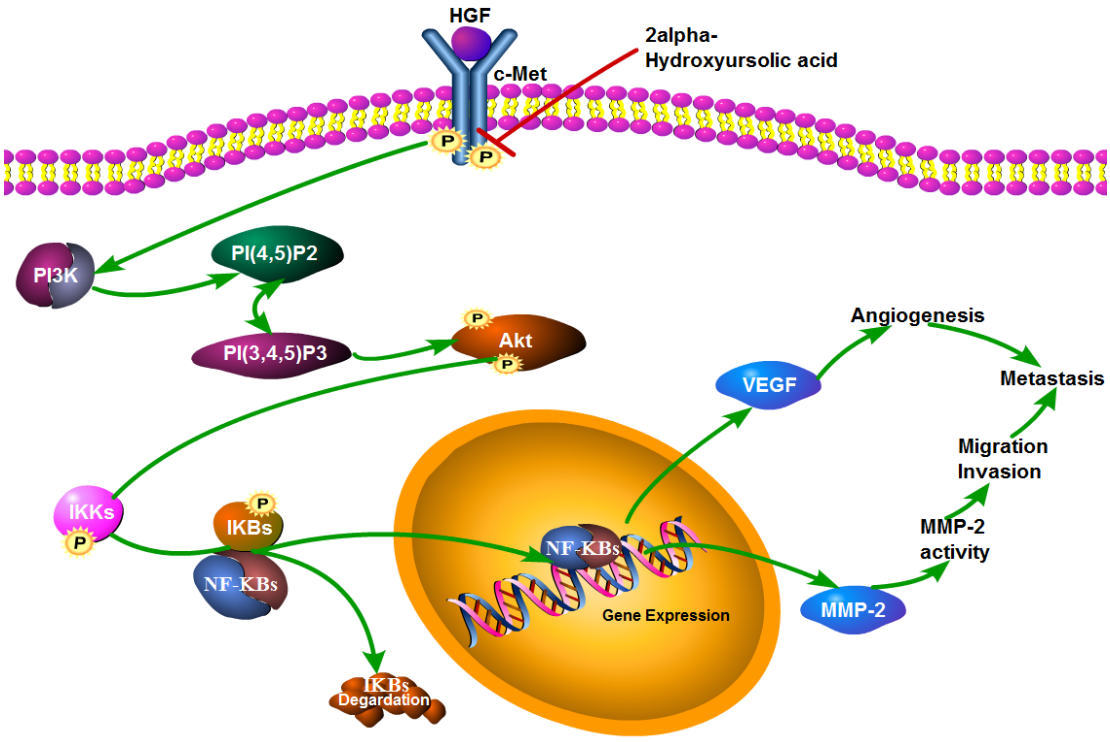

Figure 3.8. Potential mechanisms of action of 2α-hydroxyursolic acid in inhibiting cell metastasis in MDA-MB-231 human breast cancer cells through blocking HGF/Met-PI3K/Akt-NF-κB signal transduction pathway.

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CHAPTER 4

2α-HYDROXYURSOLIC ACID INHIBITED CELL METASTASIS THROUGH BLOCKING EGF/EGFR-PI3K/Akt-NF-κB/AP-1 SIGNAL TRANSDUCTION PATHWAY

Abstract

Phytochemicals in fruits and vegetables have been suggested to be responsible for their important role in the prevention of cancer. Our previous work demonstrated 2α-hydroxyursolic acid, one of the major triterpenoids isolated from apple peels, inhibited cell proliferation and induced apoptosis against human breast cancer cells. However, little is known regarding the anti-metastatic activities of 2α-hydroxyursolic acid as well as its underlying mechanism on breast cancer. The objective of this study was to investigate the effect of 2α-hydroxyursolic acid in inhibiting cell metastasis in MDA-MB-231 human breast cancer cells and its potential mechanisms of actin. The results showed that EGF stimulated cell migration and invasion instead of proliferation of MDA-MB-231 cells. 2α-Hydroxyursolic acid significantly inhibited EGF-induced MDA-MB-231 cell migration and invasion at the tested concentrations without cytotoxicity. Western blot analysis indicated that 2α-hydroxyursolic acid significantly inhibited EGFR-induced phosphorylation of EGFR and Akt, nuclear protein levels of NF-κB, c-Jun and c-Fos, as well as VEGF expression. Furthermore, the gelatinolytic activities of MMP-2 and MMP-9, critical enzymes for cancer cell migration and invasion, were dramatically inhibited in a dose-dependent manner. These results

suggested that 2α-hydroxyursolic acid exhibited anti-cancer activity through the inhibition of cell metastasis involving blocking EGF/EGFR-PI3K/Akt-NF-κB/AP-1 signal transduction pathway.

Key Words: 2α-hydroxyursolic acid, breast cancer, metastasis, migration, invasion

4.1 Introduction

Cancer is a major public health problem worldwide. It has been expected to surpass heart disease as the leading cause of death in the United States in the next few years [1, 2]. A total of 40,290 breast cancer deaths are expected to occur among US women in 2015, making breast cancer the second leading cause of cancer death among women after lung cancer [3]. Despite recent advances in tumor detection and treatment, distant metastasis remains the predominant cause of death in patients with breast cancer [4, 5].

Metastasis is the spread of cancer cells from their primary site to other parts of the body. As the tumor becomes bigger, cancer cells start competing for nutrients and oxygen to survive. In this case, angiogenic factors will be released, which stimulate endothelial cells nearby to multiply and to construct new capillaries [6]. This process is termed angiogenesis, which benefits cancer cells in two ways. First, it provides the nutrients and oxygen that needed for the surrounding cells to grow. Second, it provides the cancer cells a path to travel throughout the body to find a new site that could be distant from primary tumor [7]. Vascular endothelial growth factor (VEGF), one of angiogenic factors, proceeds to stimulate endothelial cells in the vicinity to multiply

and to construct new capillaries [8]. At the same time, carcinoma cells go through an epithelial-mesenchymal transition (EMT), which enables them to become motile and invasive. Afterwards, a metastasis starts off. Tumor cells become detached and migrate through the basement membrane and the extracellular matrix (ECM). The distinctive property that capillaries within tumors are leaky enables the cancer cells intra-vasate into them. Blood vessels transport these cancer cells to a distant site, where they may trap and subsequently extra-vasate and form dormant micrometastases (<2 mm diameter) [9, 10]. Some of the micrometastases may then reattach and colonize in the tissue where they have landed, and form a macrometastases (>2 mm diameter) by an increase in formation of new blood vessels [11]. Matrix metalloproteinases (MMPs) play important roles in this invasion-metastasis cascade-colonization process. Once activated, MMPs create spaces in ECM for cells to move and also lead to the release and activation of growth factors [8, 12].

Other than conventional therapies such as surgery and radiotherapy, molecular targeted drugs therapies are also widely used for treatment of breast cancer. Targeted agents that have been approved for breast cancer include human epidermal growth factor receptor 2 (HER2)-targeting antibody (trastuzumab and lapatinib), estrogen receptor (ER) modulator (tamoxifen) and VEGF-targeting antibody (bevacizumab) [13, 14]. Invasive breast carcinomas that were all negative for the expression of ER, progesterone receptor (PR) and HER2 (triple negative breast cancer). Unlike other subtypes, this category of breast carcinomas lacks established therapeutic targets due to limited number of receptors, making molecular targeted drugs therapies challenging [5, 15]. Increased expression of epidermal growth factor receptor (EGFR), a

transmembrane receptor, has been observed in metastatic human breast cancer cells [16]. Epidermal growth factor (EGF) was used as a chemoattractant for cancer cells [17]. Activation of EGF/EGFR pathway regulates many processes associated with metastases, which makes EGFR a potential molecular target for metastasis inhibition [18]. EGF binding to EGFR leads to auto-phosphorylation at specific tyrosine residues within the intracellular domain, which activates downstream signaling pathways, including the phosphatidylinositol-3-kinase (PI3K)/Akt pathway. PI3K/Akt pathway is known to play an important role in cell growth, proliferation, apoptosis, motility, epithelial EMT, angiogenesis and metastasis [19, 20]. Activated Akt, major downstream targets of PI3K, has been reported to promote the activation of transcription factors, such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB), resulting in activation of gene transcription, such as MMPs and VEGF [20-25]. Targeting crucial signaling pathways and metastasis-related molecules would be a promising adjuvant strategy to inhibit the migration and invasion of highly metastatic cancer cells.

Epidemiological studies showed that diets rich in whole grains, vegetables, and fruits, which contain high levels of phytochemicals, have provided a significant inverse association with breast cancer [26-32]. 2α-Hydroxyursolic acid, isolated from apple peels previously by our lab [33], also has been discovered in other fruits, vegetables [34-36] and traditional Chinese medicinal herbs.[37-40] Previous research reported that 2α-hydroxyursolic acid displays anti-cancer effect against several human cancer cell lines, including lung adenocarcinoma A549 cells [41], human gastric carcinoma SNU-601 cells [42], human promyelocytic leukemia HL-60 cells [43] and

human breast cancer MCF-7 cells [44]. We previously reported that 2α-hydroxyursolic acid inhibited cell proliferation and induced apoptosis in MDA-MB-231 human breast cancer cells [45]. However, little is known regarding the anti-metastatic activities of 2α-hydroxyursolic acid as well as its underlying mechanisms of action on breast cancer. The purpose of this study was to examine the mechanisms of action of 2α-hydroxyursolic acid in inhibiting cell metastasis in a highly metastatic breast cancer cell line, MDA-MB-231 human breast cancer cells.

4.2 Materials and Methods

4.2.1 Cell culture and chemicals

MDA-MB-231 human breast cancer cells purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained at 37 °C in a humidified 5% CO_2 incubator in α-MEM containing 1% antibiotic-antimycotic, 10% heat-inactivated fetal bovine serum 10 mM PH=7.4 Hepes as described previously [44, 46]. 2α-Hydroxyursolic acid was isolated from apple peels by our lab as described previously [33]. Dimethyl sulfoxide (DMSO) was purchased from VWR (Radnor, PA, USA). Phosphate-buffered saline (PBS) and α-Minimum Essential Medium (α-MEM) were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). Methylene blue was purchased from BBL (Cockeysville, MD, USA). Polyoxyethylene (tween 20) sorbitan monolaurate was obtained from Calbiochem (Billerica, MA, USA). Hepes, Folin-Ciocalteu reagent, protease inhibitors (aprotinin; leupeptin; pepstain; sodium

orthovanadate), Epidermal Growth Factor (EGF), gelatin, IGEPAL (CA-630) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Primary antibody against p-EGFR, EGFR, p-Akt, Akt, NF-κB, c-Jun, c-Fos, VEGF and Nucleolin were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against β-actin, anti-mouse IgG and anti-rabbit IgG were obtained from Sigma-Aldrich Inc. (St. Louis, MO).

4.2.2 Assessment of anti-proliferative activity by methylene blue assay

The anti-proliferative activity of 2α-hydroxyursolic acid was measured by the methylene blue assay described previously [47]. MDA-MB-231 human breast cancer cells in 100 µL fresh complete medium were plated in 96-well plate at a density of 2.5 × 10^4 cells/ well and were incubated at 37 °C in 5% CO_2 for 8 h. Then starve the cells in the serum free medium (SFM) overnight. Next, the cells were treated by various concentrations of EGF (1 ng/mL, 10 ng/mL and 100 ng/mL) with or without 20 µM 2α-hydroxyursolic acid as samples or 1% DMSO only as control in 100 µL fresh medium. After additional 72 hours incubation, the growth medium was removed from each well and the cells were washed with 100 µL phosphate-buffered saline (PBS). Cells were then stained with methylene blue solution [98% Hanks Balanced Salt Solution (HBSS), 0.67% glutaraldehyde, 0.6% methylene blue] and incubated for 1 hour. Next, the solution was removed and rinsed with deionized water three times. After the wells were air-dry, methylene blue stain in cells was eluted with the elution buffer [1% (v/v) acetic acid, 49% (v/v) PBS, and 50% (v/v) ethanol] by rotating on a

bench shaker for 20 minutes. The absorbance was read at 570 nm by using FilterMax F5 Multi-Mode Microplate Readers (Molecular Devices, Sunnyvale, CA). The anti-proliferative activity was determined as percentage compared to the control. All measurements were conducted in triplicate.

4.2.3 Wound-healing assay

MDA-MB-231 cells were seeded in 6-well plate at a density of 5.0×10^5 cells/ well and were incubated in complete medium at 37 °C in 5% CO_2 for 24 h. The growth medium was then replaced with 1.5 mL SFM to starve the cells for another 24 h. The wounds were made using a 200 µL disposable plastic pipette tip (approximately 1 mm in size) and washed with PBS twice. The cells were soon treated with either SFM or EGF (100 ng/mL) in the presence of 2α-hydroxyursolic acid at doses of 0, 15, 20 and 25 µM. The wounds were observed using bright field microscopy. Pictures were taken at three randomly selected distinct view fields flanking the intersections of the wound and the marker lines made by a needle at the very beginning three times at 0, 12, and 24 h. Data were analyzed using Sigmaplot software version 11.0 (Systat Software, Inc. Chicago, IL). The migration rate was measured by the percentage of wound area at 12 and 24 h versus the origin area at 0 h. The area is analyzed by Adobe Photoshop CS4 (Adobe systems Inc. California, USA). Results will be conducted in triplicate.

4.2.4 Cell invasion assay

The CHEMICON Cell Invasion Assay Kit, purchased from EMD Millipore (Merck

KGaA, Darmstadt, Germany), was used to detect cell invading activity. After the extracellular matrix (ECM) layer being activated by the warm medium, 500 µl of SFM containing 100 ng/mL EGF was added to the lower chamber and MDA-MB-231 breast cancer cells (5.0 x 10^5 cells/mL) in 300 µl SFM with different concentrations of 2α-hydroxyursolic acid (0, 15, 20 and 25 µM) were added to each inserts. After 24 hours incubation at 37°C in 5% CO_2, non-invading cells and the ECMatrix gel were gently removed using a cotton-tipped swab from the interior of the inserts. The inserts were dipped in the staining solution for 30 minutes to stain the invading on lower surface of the membrane. Dip inserts in a beaker of water several times to wash off the redundant staining solution and allow the inserts to dry. Quantitate the cells by dissolving stained cells in 10% acetic acid (100-200 µl/well) and transfer a consistent amount of the dye/solute mixture to a 96-well plate for colorimetric reading of OD at 560 nm. All measurements were conducted in three replications.

4.2.5 Gelatin zymography assay

Gelatin zymography, a widely used technique in the study of matrix metalloproteases (MMPs), is described previously [48]. MDA-MB-231 cells were seeded in 6-well plate at a density of 1.0 × 10^6 cells/ well and were incubated in complete medium at 37 °C in 5% CO_2 for 24 h. Then the complete medium was replaced with 1.5 mL SFM to starve the cells for another 24 h. The SFM were removed and the cells were treated with either SFM or EGF (100 ng/mL) in the presence of 2α-hydroxyursolic acid at doses of 0, 15, 20 and 25 µM. The medium was harvest after 24 h and centrifuged for

3 min at 400 g. Collected supernatant was then mixed with 2% SDS loading buffer and subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis with 0.8 mg/mL gelatin. After running at 150 V for roughly 1 h, the gel was gently washed using fresh enzyme renaturing buffer, which contains 2.5% (v/v) Triton X-100, 15 min for 4 times, to remove SDS. To allow proteolysis of the gelatin substrate, the gel was transferred to developing buffer (enzyme renaturing buffer without Triton X-100) agitated on shaker for 15 min and followed by incubating in developing buffer for 20 h at 37 ℃. Then stain the gel with 0.125% (w/v) Coomassie brilliant blue in 20% (v/v) acetic acid and 50% (v/v) methanol, followed by destaining in 30% (v/v) methanol and 1% (v/v) formic acid. Clear bands of gelatinolytic activity were visualized and photographed by digital camera and quantified by ImageJ2x software (Wayne Rasband, National Institutes of Health, Maryland, USA). The MMPs activity was determined as percentage compared to control. All measurements were conducted in three replications.

4.2.6 Preparation of whole-cell lysates and nuclear fraction

Whole-cell lysates and nuclear fractions of cells were prepared by using the method as reported previously in our laboratory [49]. Briefly, MDA-MB-231 human breast cancer cells were seeded at a density of 5.0×10^5 cells/ well in 6-well plates. After 8 hours incubation at 37°C in 5% CO_2 and then the cells were incubated overnight without serum. Next, various concentrations of 2α-hydroxyursolic acid (0, 15, 20 and 25 μM) were added into the wells for 2 h at 37 °C in 5% CO_2 incubation, the cells

were stimulated by 100 ng/mL EGF for 30 min. Then the cells were washed twice with ice-cold PBS, scraped off from the wells and centrifuged for 5 min at 400 g. For whole-cell lysates, harvested cells were then lysed using lysis buffer (50 mM Tris, pH 7.4; 1% Igepal; 150 mM sodium chloride; 1 mM EDTA) with different protease inhibitors (1 g/mL aprotinin; 1 g/mL leupeptin; 1 g/mL pepstain; 1 mM sodium orthovanadate). Cell lysates were vortexed briefly every 5 min for 30 min totally to facilitate protein extraction. Lysates were then centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was collected as whole-cell lysates. For nuclear fraction, the harvested cells were added buffer A (10 mM HEPES pH 7.8, 10 mM KCl, 0.1 mM EDTA) with reducing agent 1mM DTT, and protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin and 0.2 mM sodium orthovanadate). Cells were kept on ice 15 min and the cell suspension was mixed for 15 s after 1% IGEPAL (CA-630) was added. The cell suspension was centrifuged at 2000 g for 3 min at 4 °C. The supernatant was discarded and the deposit in buffer B (50 mM HEPES-KOH pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) with 1mM DTT and protease inhibitors, then vortexed every 2-3 min. Nuclear lysates were centrifuged at 12,000 g for 30 min at 4 °C and the supernatant was collected as the nuclear extract.

4.2.7 Western blot assay

The western blot analysis was conducted as described previously [49, 50]. Protein concentrations of both whole-cell lysates and nuclear fractions were determined using a Sigma Diagnostics Micro Protein Determination Kit and a FilterMax F5 Multi-Mode

Microplate Readers (Molecular Devices, Sunnyvale, CA). Equal amounts of protein from each cell lysate was subjected to 10% SDS-PAGE, running for roughly 2 h and then transferred onto PVDF membranes. The membranes were blocked for 2 h in TBST (Tris-base buffer solution containing of 0.1% Tween 20) containing 5% non-fat dry milk at room temperature. Then the membranes were incubated with the desired primary antibody overnight at 4˚C and secondary antibody for 2 h at room temperature. Membrane-bound antibodies were stimulated by the Enhanced Chemiluminescence kit (Cell Signaling Technology, Inc., Beverly, MA) and sensed by Kodak Biomax MR Film (Kodak, Rochester, NY) after developing and fixing procedures. Bands were then scanned and quantified by ImageJ2x software (Wayne Rasband, National Institutes of Health, Maryland, USA). The expression of human α-tubulin or β-actin was used as an internal standard control. All measurements were conducted in triplicate.

4.2.8 Statistical analysis

Data were analyzed using Sigmaplot software version 11.0 (Systat Software, Inc. Chicago, IL) and dose-effect analysis was performed using Calcusyn software version 2.0 (Biosoft, Cambridge, UK). Data were presented as mean ± SD for at least three independently performed experiments. Statistical analyses were carried out with Tukey's test and analysis of variance (ANOVA) by JMP software version 9.0.2 (SAS Institute Inc. North Carolina, USA). Differences with $p<0.05$ were considered to be statistically significant.

4.3 Results

4.3.1 EGFR pathway is not involved in the inhibitory effect of 2α-hydroxyursolic acid on proliferation of MDA-MB-231 human breast cancer cells

2α-Hydroxyursolic acid significantly inhibited proliferation of MDA-MB-231 human breast cancer cells in a dose-dependent manner as described previously by our lab [45]. To elaborate the role of EGFR in 2α-hydroxyursolic acid inhibited cell proliferation, we tested the effect of EGFR ligand, EGF, on proliferation in MDA-MB-231 human breast cancer cells. After starvation, MDA-MB-231 human breast cancer cells were treated by various concentrations of EGF (1, 10 and 100 ng/mL) with or without 20 μM 2α-hydroxyursolic acid. Treatment of MDA-MB-231 cells with different concentrations of EGF alone did not induce cell proliferation (Figure 4.1). Treatment with 20 μM of 2α-hydroxyursolic acid inhibited the proliferation of MDA-MB-231 human breast cancer cells by 56.79%. However, addition of 100 ng/mL EGF did not abolish the anti-proliferative effect of 2α-hydroxyursolic acid in treated MDA-MB-231 human breast cancer cells (Figure 4.1).

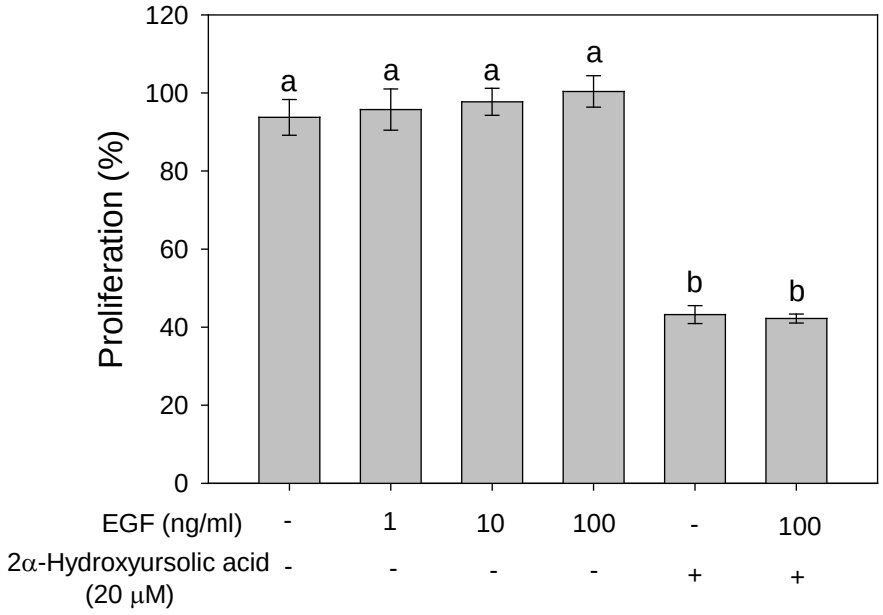


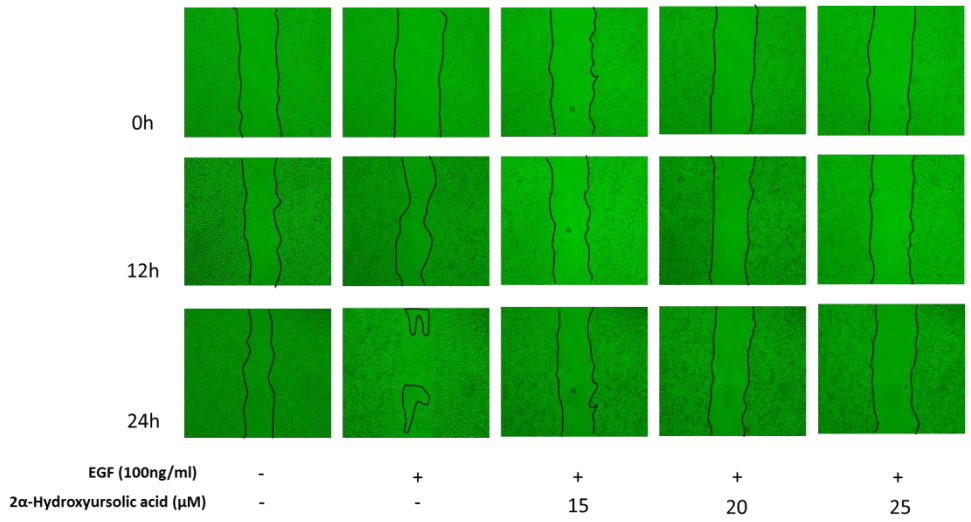
Figure 4.1. Effect of EGF and 2α-hydroxyursolic acid on cell proliferation in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

4.3.2 Inhibitory effect of 2α-hydroxyursolic acid on EGF-induced migration in MDA-MB-231 human breast cancer cells

There is striking resemblance between tumor progression and wound healing [51]. In wound healing, epithelial cells also need to go through EMT, which enables these cells to move into the wound site. To evaluate whether 2α-hydroxyursolic acid affected EGF-mediated cell migration, wound-healing migration assay was performed. After starvation, cells were cultured in either SFM or EGF (100 ng/mL) in the presence of different doses of 2α-hydroxyursolic acid (0, 15, 20 and 25 μM). The EGF-treated

cells migrated into the cell-free area were far more than the starved untreated MDA-MB-231 cells at 12 h and 24 h after scratched the monolayers (Figure 4.2A). The stimulatory effect of EGF was significantly attenuated in the presence of 2α-hydroxyursolic acid. As shown in Figure 4.2B, the wound width is 64.88, 80.04 and 91.52% after treated with 15, 20 and 25 μM 2α-hydroxyursolic acid for 24 h, compared with only 33.24% wound width in EGF control group for 24 h. The results demonstrated that the EGF-induced MDA-MB-231 cell migration was inhibited by 2α-hydroxyursolic acid in a dose-dependent manner (Figure 4.2, $p<0.05$).

A



B

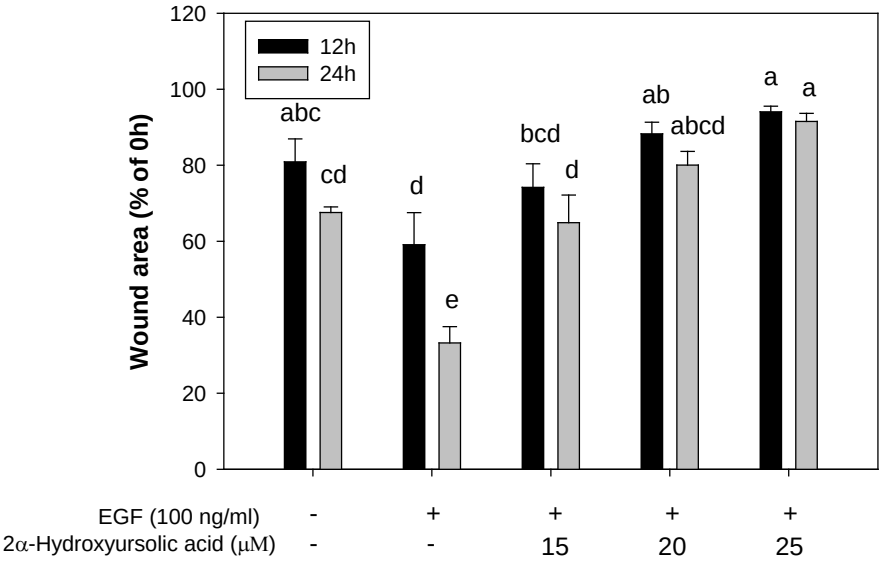


Figure 4.2. Effect of 2α-hydroxyursolic acid on the cell migration in MDA-MB-231 human breast cancer cells. In the wound-healing assay, the serum-starved monolayers

cells were wounded using a disposable plastic pipette tip and the cells were treated with either SFM or EGF (100 ng/mL) in the presence of 2α-hydroxyursolic acid at doses of 0, 15, 20 and 25 μM. (A) Width of wounds was observed using bright field microscopy and the representative photographs showed the same area three times per well at 0, 12, and 24 h. (B) The migration rate was measured by the percentage of wound area at 12 and 24 h versus the origin area at 0 h. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

4.3.3 Inhibitory effect of 2α-hydroxyursolic acid on EGF-induced invasion in MDA-MB-231 human breast cancer cells

After we observed the inhibitory effect of 2α-hydroxyursolic acid on the EGF-mediated migration in MDA-MB-231 human breast cancer cells, we then decided to determine the effect of 2α-hydroxyursolic acid on EGF-induced invasion using the Transwell chamber assay. ECMatrix, proteins derived from the Engelbreth Holm-Swarm (EHS) mouse tumor was used as basement membrane matrix in this assay to evaluate the invasive ability of tumor cells [52]. Compared to the starved untreated MDA-MB-231 cells, EGF treatment at 100 ng/mL significantly increased cell invasion as indicated 150% more cells passing through the transwell chamber polycarbonate membrane (Figure 4.3). However, treatment with 15, 20 and 25 μM 2α-hydroxyursolic acid remarkably suppressed EGF-induced cell invasion by 21.13, 54.83 and 66.52% (Figure 4.3, $p<0.05$)

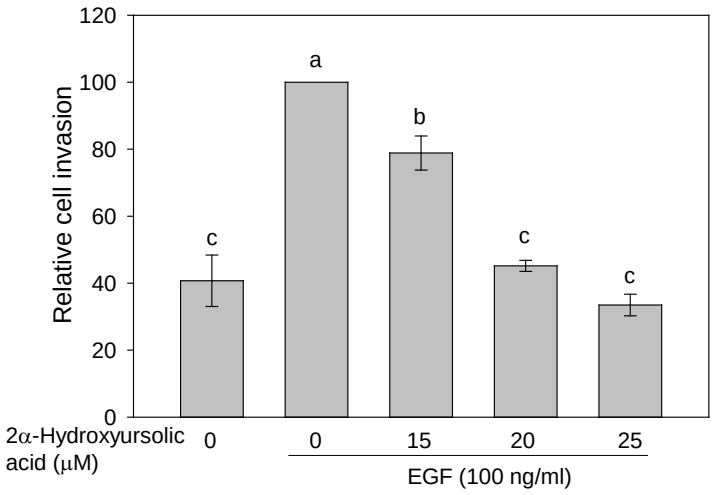


Figure 4.3. Effect of 2α-hydroxyursolic acid on the cell invasion in MDA-MB-231 human breast cancer cells. In the cell invasion assay, SFM containing MDA-MB-231 breast cancer cells and different concentrations of 2α-hydroxyursolic acid (0, 15, 20 and 25 μM) were added into the inserts. Either SFM or EGF (100 ng/mL) was applied to the lower chamber as a chemoattractive agent. The invading cells on the lower surface of the membrane filter were stained by stain solution after 24 h. (A) Representative photographs showed the stained invading cells under different treatment. (B) The invasion rate was measured by the colorimetric reading of OD at 560 nm after the staining and stain dissolving steps. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

4.3.4 Inhibitory effect of 2α-hydroxyursolic acid on EGF-induced MMP-2 and

MMP-9 activities in MDA-MB-231 human breast cancer cells

To determine the effects of 2α-hydroxyursolic acid on EGF-induced MMP-2 and MMP-9 activities, gelatin zymography was performed in MDA-MB-231 human breast cancer cells. EGF significantly induced MMP-2 gelatinolytic activity when compared to the control. In comparison with MMP-2, relatively low level of MMP-9 induced by EGF in MDA-MB-231 human breast cancer cells (Figure 4.4A). The results showed that 2α-hydroxyursolic acid was markedly effective in inhibiting the gelatinolytic activity of MMP-2 and MMP-9 (Figure 4.4A). The MMP-2 and MMP-9 activities were significantly reduced by 64.89% and 67.09% compared to EGF control group at the dose of 25 μM, respectively (Figure 4.4B, $p<0.05$).

A

B

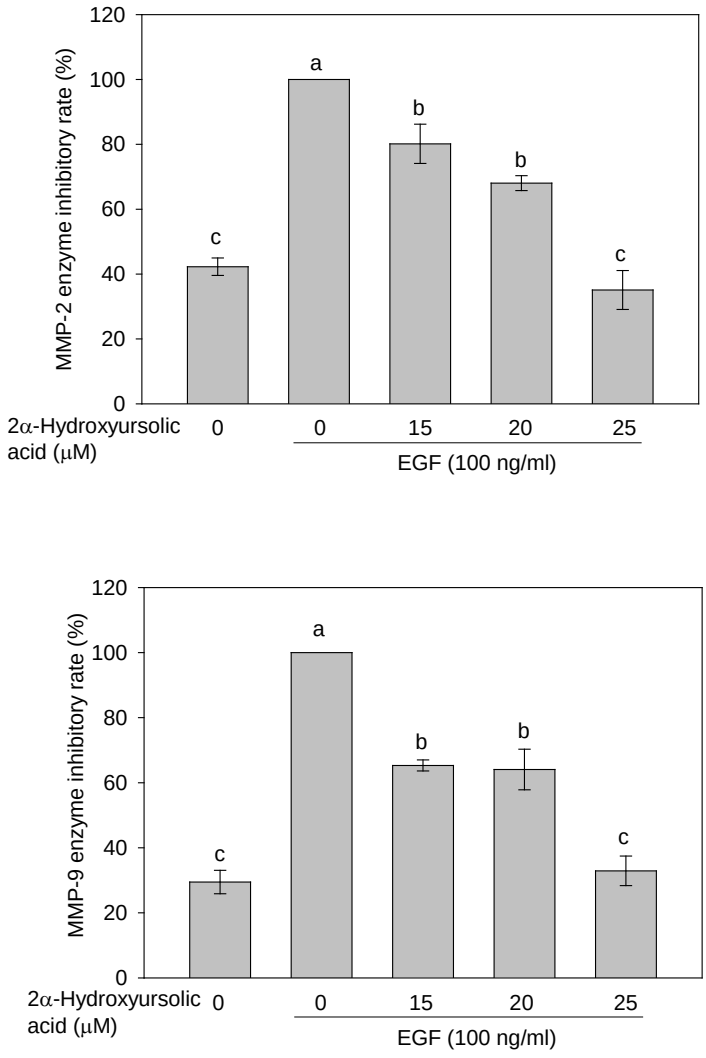


Figure 4.4. Effects of 2α-hydroxyursolic acid on the proteolytic activities of MMP-2 in MDA-MB-231 human breast cancer cells. The serum-starved cells were exposed to SFM or EGF (100 ng/mL) in the presence of 2α-hydroxyursolic acid at doses of 0, 15, 20 and 25 μM. (A) After 24 h, the medium were collected, and electrophoresed on gelatin gels and stained. Bands were then photographed by digital camera. (B) Bands were quantified by ImageJ2x software and the MMP-2 activity was determined as the

percentage compared to control. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

4.3.5 Inhibitory effect of 2α-hydroxyursolic acid on EGF-induced VEGF expression in MDA-MB-231 human breast cancer cells

To investigate the potential inhibitory effect of 2α-hydroxyursolic acid on angiogenesis in MDA-MB-231 human breast cancer cells, the expression of VEGF, an angiogenic factor, was analyzed by Western blot assay. As shown in Figure 4.5, EGF stimulated the expression of VEGF when compared to the control, whereas 2α-hydroxyursolic acid significantly down-regulated EGF-induced VEGF in MDA-MB-231 human breast cancer cells in a dose-dependent manner ($p<0.05$). At the concentration of 25 uM, 2α-hydroxyursolic acid significantly inhibited EGF-induced VEGF expression in MDA-MB-231 human breast cancer cells by 62.44% (Figure 4.5).

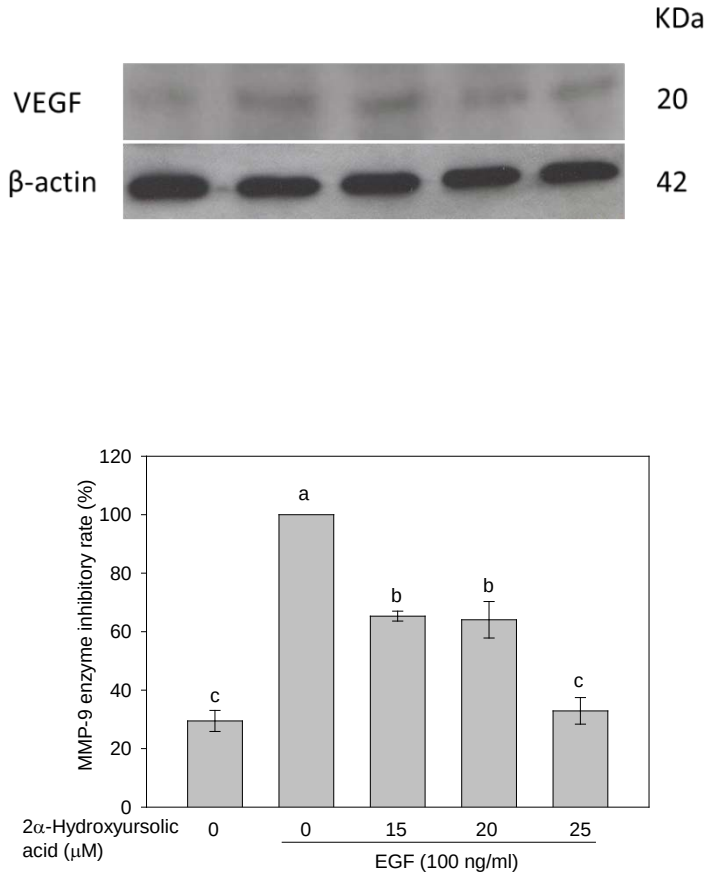


Figure 4.5. Effects of 2α-hydroxyursolic acid on expression of VEGF in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

4.3.6 Inhibitory effect of 2α-hydroxyursolic acid on EGF-induced expression of transcription factors NF-κB and AP-1 in MDA-MB-231 human breast cancer cells

In order to clarify the involvement of NF-κB and AP-1 in the mechanism of the inhibitory effect of 2α-hydroxyursolic acid in cell metastasis, the expression of NF-κB

and AP-1 family members: c-Fos and c-Jun in the nuclear extracts were analyzed by Western blot assay. As shown in Figure 4.6A and 4.7A, EGF significantly induced expression of nuclear NF-κB, c-Fos and c-Jun when compared to the control. 2α-Hydroxyursolic acid significantly suppressed the expression of nuclear NF-κB, c-Fos and c-Jun in a dose-dependent manner. Protein expression of the nuclear NF-κB, c-Fos and c-Jun were inhibited by 78.58%, 60.47% and 72.08%, respectively, in MDA-MB-231 human breast cancer cells when treated with 2α-hydroxyursolic acid at the dose of 25 μM compared to EGF-induced control group (Figure 4.6 and 4.7, $p<0.05$).

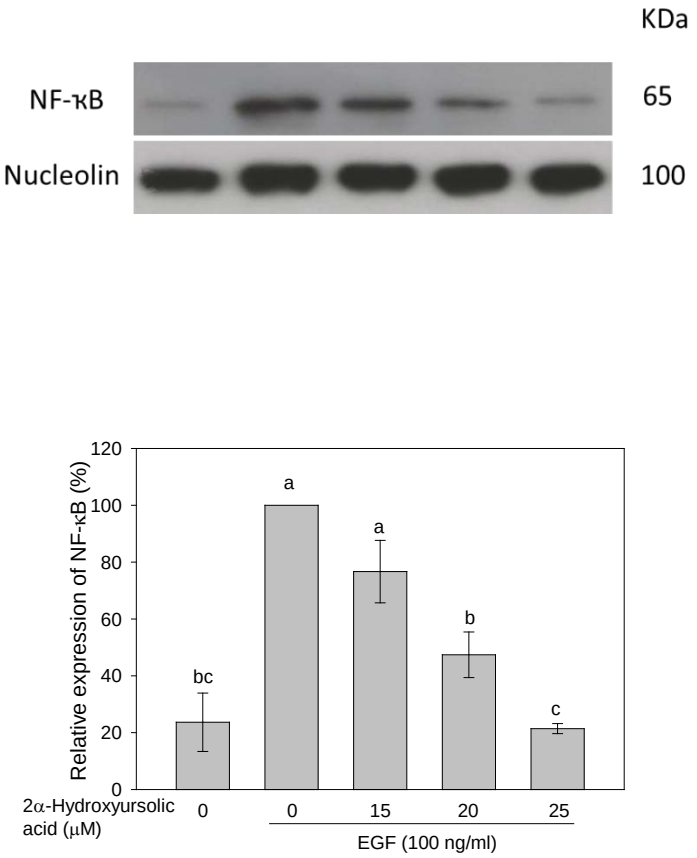
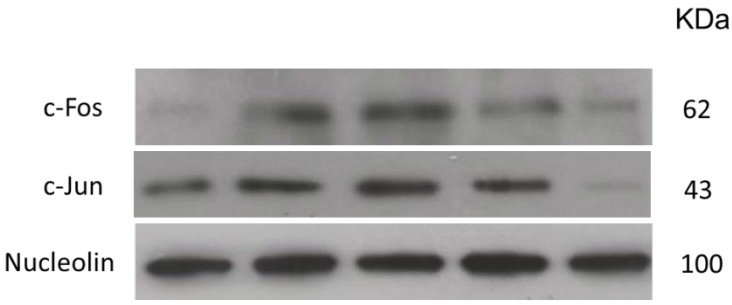
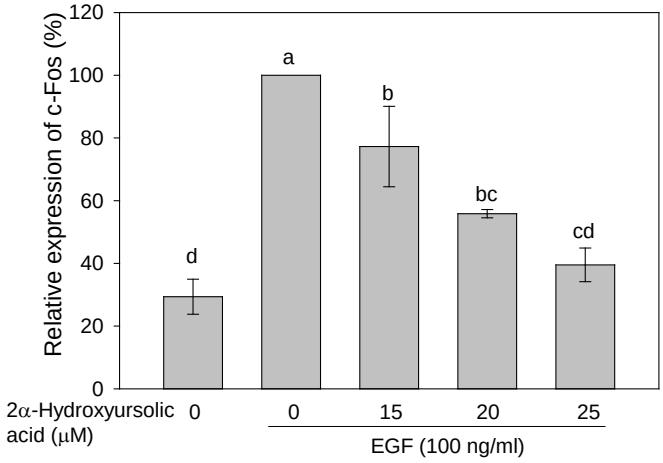


Figure 4.6. Effects of 2α-hydroxyursolic acid on expression of nuclear NF-κB in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.


KDa
c-Fos
c-Jun
Nucleolin
62
43
100


Relative expression of c-Fos (%)
120
100
80
60
40
20
0
a
b
bc
cd
d
2α-Hydroxyursolic acid (μM)
0
0
15
20
25
EGF (100 ng/ml)

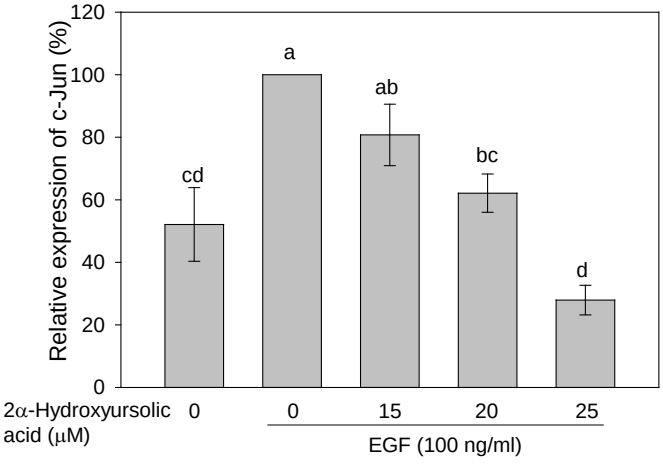
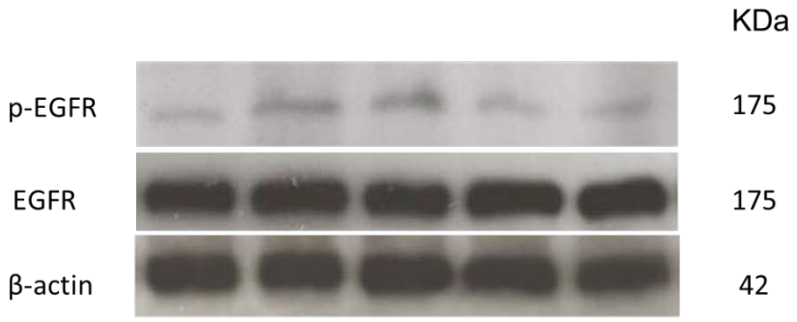
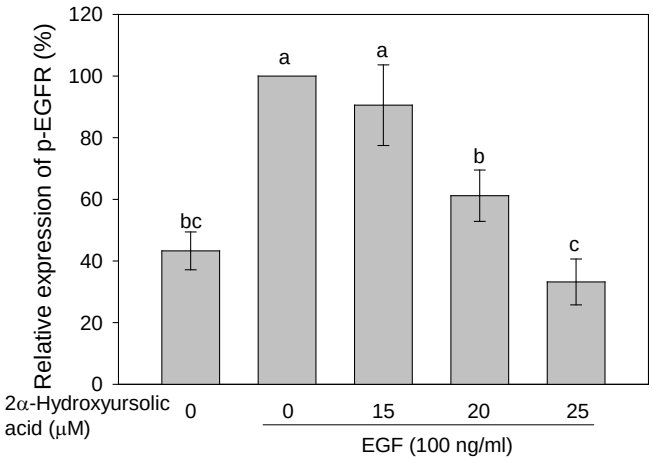

Relative expression of c-Jun (%)
120
100
80
60
40
20
0
a
ab
bc
cd
d
2α-Hydroxyursolic acid (μM)
0
0
15
20
25
EGF (100 ng/ml)

Figure 4.7. Effects of 2α-hydroxyursolic acid on expression of nuclear c-Jun and c-Fos in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p< 0.05$). Each value represents the mean ± SD of triplicates.

4.3.7 Inhibitory effect of 2α-hydroxyursolic acid on EGF-induced phospho-EGFR in MDA-MB-231 human breast cancer cells

In order to further elaborate the molecular mechanisms of how 2α-hydroxyursolic acid inhibits EGF-mediated metastasis in MDA-MB-231 cells, we measured the effect of 2α-hydroxyursolic acid on EGFR phosphorylation. The result showed that 2α-hydroxyursolic acid significantly inhibited the EGF-induced phosphorylation of EGFR in a dose-dependent manner. In the group receiving 25 µM of 2α-hydroxyursolic acid, the phosphorylation of EGFR was inhibited by 66.81% when compared to the EGF induced control (Figure 4.8, $p<0.05$). The total protein levels of EGFR remained unchanged with these treatments (Figure 4.8).


KDa
p-EGFR
175
EGFR
175
β-actin
42


Relative expression of p-EGFR (%)
120
100
80
60
40
20
0
a
a
b
bc
c
2α-Hydroxyursolic acid (μM)
0
0
15
20
25
EGF (100 ng/ml)

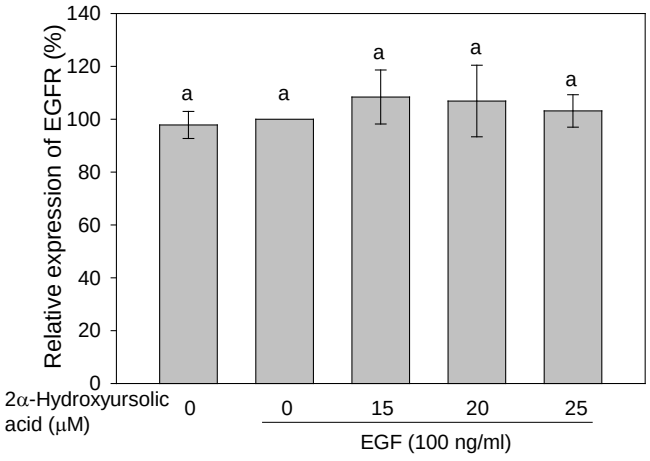
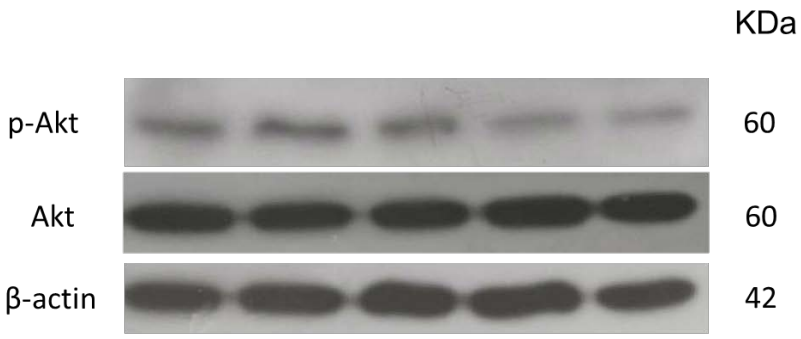
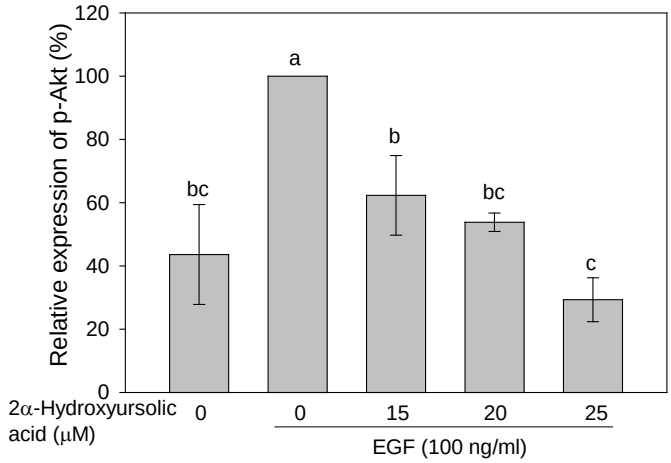

Relative expression of EGFR (%)
140
120
100
80
60
40
20
0
a
a
a
a
a
2α-Hydroxyursolic acid (μM)
0
0
15
20
25
EGF (100 ng/ml)

Figure 4.8. Effects of 2α-hydroxyursolic acid on expression of p-EGFR and EGFR in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

4.3.8 Inhibitory effect of 2α-hydroxyursolic acid on EGF-induced PI3K/Akt in MDA-MB-231 human breast cancer cells

Akt, major downstream targets of PI3K, is a multifunctional regulator of cell survival, growth and invasion [21, 22]. In order to determine the effect of 2α-hydroxyursolic acid on Akt phosphorylation, western blot analysis was performed. As shown in Figure 4.9, EGF significantly induced the phosphorylation of Akt when compared to the control. However, the EGF-induced phosphorylation of Akt was inhibited by 2α-hydroxyursolic acid in a dose-dependent manner while total protein levels of Akt remained unchanged when cells were treated with various concentrations of 2α-hydroxyursolic acid ($p<0.05$). In the group receiving 25 μM of 2α-hydroxyursolic acid, the phosphorylation of Akt was inhibited by 70.68% when compared to the EGF induced control (Figure 4.9, $p<0.05$).


KDa
p-Akt
60
Akt
60
β-actin
42


Relative expression of p-Akt (%)
120
100
80
60
40
20
0
a
b
bc
bc
c
2α-Hydroxyursolic acid (μM)
0
0
15
20
25
EGF (100 ng/ml)

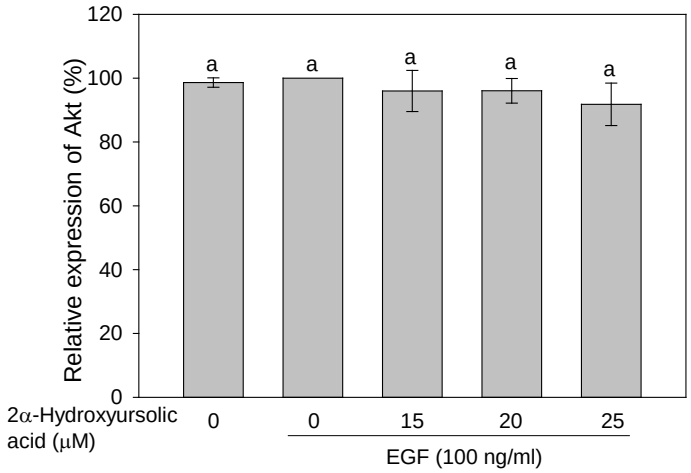

Relative expression of Akt (%)
120
100
80
60
40
20
0
a
a
a
a
a
2α-Hydroxyursolic acid (μM)
0
0
15
20
25
EGF (100 ng/ml)

Figure 4.9. Effects of 2α-hydroxyursolic acid on expression of p-Akt and Akt in MDA-MB-231 human breast cancer cells. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

4.4 Discussion

Approximately 50% of women with breast cancer will develop metastatic disease. Cancer metastasis occurs by a complex series of events: epithelial-mesenchymal transition (EMT), migration, invasion, induction of angiogenesis, and growth at metastatic organs such as lung, liver and bone [5, 53, 54]. It is estimated that one third of all cancer mortality in the United States could be avoided through changing in dietary behavior [55-57]. The 2015-2020 Dietary Guidelines for Americans recommend that people should consume a variety of vegetables from all of the subgroups, fruits (especially whole fruits) and grains (at least half of which are whole grains) in order to keep a healthy eating pattern [58]. Phytochemicals, defined as bioactive non-nutrient plant chemicals in fruits, vegetables, whole grains, and other plant-based foods may provide desirable health benefits beyond basic nutrition to reduce the risk of the development of chronic diseases [59, 60]. Apple is a commonly eaten fruit in the United States. Apple phytochemical extracts showed anti-proliferative activities against MCF-7 and MDA-MB-231 human breast cancer cell, as well as prevent mammary cancers formation in an animal study [46, 61]. However, unpeeled apple exhibited even higher antioxidant activity and anti-proliferative

activity compared to peeled apples [62]. The main bioactive components in apple peels are phenolic compounds and triterpenes [33, 63].

2α-Hydroxyursolic acid, one of major active triterpenoids isolated from apple peels, has attracted great attention regarding its potential as an anti-cancer agent in various types of cancer cells.[23, 38-41] Horlad et al. [64] reported that orally administrated to mice with 2α-hydroxyursolic acid before and after subcutaneous tumor implantation with LM85 cells suppressed lung metastasis. However, the ability of 2α-hydroxyursolic acid in inhibiting invasion, migration, and angiogenesis in breast cancer cells has not been reported, and little is known about the mechanisms of action account for these effects. In this study, we report that 2α-hydroxyursolic acid inhibited proliferation of MDA-MB-231 human breast cancer cells, but not through EGF/EGFR pathway. 2α-hydroxyursolic acid significantly inhibited EGF-induced MDA-MB-231 human breast cancer cells migration, invasion, MMP-2, MMP-9 activity and VEGF expression. No cytotoxicity was observed under the tested concentrations [45]. In addition, 2α-Hydroxyursolic acid remarkably reduced EGF-induced EGFR phosphorylation, Akt phosphorylation, nuclear NF-κB and AP-1 in dose-dependent manners when compared to the EGF-induced control group.

4.4.1 EGF/EGFR pathway is involved in the inhibitory effect of 2α-hydroxyursolic acid on cell migration and invasion instead of proliferation of MDA-MB-231 human breast cancer cells

We reported previously that 2α-hydroxyursolic acid significantly inhibited MDA-MB-

231 human breast cancer cells proliferation in a dose-dependent manner and no cytotoxicity was observed at the concentration below 30 μM [45]. Although EGF is well known being a potently mitogenic for many normal and tumor cells, a lack of a proliferative response to EGF of MDA-MB-231 human breast cancer cells was observed in the present study. The result showed that treatment of MDA-MB-231 cells with different concentrations of EGF (1, 10 and 100 ng/mL) alone did not induce cell proliferation (Figure 4.1). This finding is consistent with previous works by Davidson et al. [65] and Price et al. [66], who reported MDA-MB-231 human breast cancer cells express EGFR but lack a proliferative response to EGF. Therefore, we concluded that EGF/EGFR pathway is not involved in the inhibitory effect of 2α-hydroxyursolic acid on proliferation of MDA-MB-231 human breast cancer cells. Next, we decided to determine the effect of 2α-hydroxyursolic acid in EGF-mediated metastasis. Since cell migration and invasion activity were considered to be the vital steps for metastatic tumor cells, wound healing assay and cell invasion assay were employed to evaluate the anti-metastatic potential of 2α-hydroxyursolic acid. The results showed that 2α-hydroxyursolic acid inhibited EGF-induced migration and invasion of MDA-MB-231 human breast cancer cells in a dose-dependent manner at non-cytotoxic concentrations tested (Figure 4.2 and 4.3).

4.4.2 2α-Hydroxyursolic acid inhibited the EGF-induced MMP-2, MMP-9 activity and VEGF expression in MDA-MB-231 human breast cancer cells

There are molecules playing important roles in cancer metastasis, including matrix metalloproteases (MMPs) and vascular endothelial growth factor (VEGF) [5, 48]. Degradation of ECM and the basement membrane is required to allow cell migration and invasion processes to occur. MMPs, a family of zinc-binding endopeptidases, are key enzymes that participate in the proteolytic destruction of environmental barriers, such as ECM and basement membranes [67, 68]. Two members of the MMP family, MMP-2 (gelatinase A or 72-kDa type IV collagenase) and MMP-9 (gelatinase B or 92-kDa type IV collagenase), have been shown to be highly expressed and correlated with poor survival in patients with breast cancer [69, 70]. VEGF, an important angiogenic factor, is a mitogen specific for endothelial cells. It helps develop new blood vessels for cancer cells to travel throughout the body through inducing the proliferation of vascular endothelial cells [71, 72]. Therefore, in order to identify whether 2α-hydroxyursolic acid influences MMP-2, MMP-9 gelatinolytic activity and VEGF expression, wound-healing assay and western blot assay are employed. The results showed that 2α-hydroxyursolic acid significantly suppress MMP-2, MMP-9 activity and VEGF expression in a dose-dependent manner (Figure 4.4 and 4.5)

4.4.3 2α-Hydroxyursolic acid inhibited the EGF/EGFR, PI3K/Akt, NF-κB and AP-1 pathways in MDA-MB-231 human breast cancer cells

Aberrations in EGFR expression and downstream intracellular-signaling cascades, including the PI3K/Akt, contribute to the progression, invasion, migration and angiogenesis in many human cancers, including breast cancer [6, 73, 74]. The AP-1 and NF-κB transcription factor have to be reported to play essential role in a wide variety of biological responses including EMT, cell invasion and motility [24, 75]. Several angiogenic factors, including VEGF, are also promoted by NF-κB and AP-1 [76, 77]. NF-κB complex is sequestered as an inactive form in the cytoplasm by inhibitory IκB proteins. Once phosphorylated by IκB kinase (IKK), IκB undergoes ubiquitin-dependent degradation by the proteasome, resulting in the translocation of NF-κB into nucleus [78]. NF-κB then binds to the promoter region of target genes that control cancer cell invasion, metastasis, and angiogenesis (such as MMPs and VEGF) to activate gene expressions [76, 79, 80]. AP-1 is a dimeric transcription factor that composed of members of the Fos and Jun proto-oncogene families [17]. Thus, western blot assay is conducted to investigate if 2α-hydroxyursolic acid could interfere with these crucial signaling pathways, including EGF/EGFR, PI3K/Akt, NF-κB and AP-1. 2α-Hydroxyursolic acid significantly down-regulated EGF-induced phosphorylation of EGFR and Akt (Figure 4.8 and 4.9), as well as nuclear NF-κB, c-Jun and c-Fos transcription factor (Figure 4.6 and 4.7). While the total protein levels of EGFR and Akt remained unchanged with these treatments (Figure 4.8 and 4.9). No research has been reported to investigate the molecular interaction between 2α-hydroxyursolic acid

and EGFR. EGFRs are transmembrane receptors composed of an extracellular binding component, a transmembrane component and an intracellular tyrosine kinase component. Monoclonal antibodies bind to the extracellular domain, while small-molecule inhibitors such as gefitinib and Lapatinib bind to the tyrosine kinase domain and stop the activity of the EGFR [81]. Sathya et al. has reported that ursolic acid inhibit the phosphorylation of EGFR by binding with the adenosine triphosphate (ATP) binding pocket of EGFR kinase domain. As a derivative of ursolic acid, it is possible that 2α-hydroxyursolic acid inhibit receptor signal processes by binding to the ATP-binding pocket of the EGFR protein kinase domain.

In conclusion, our data demonstrate the first time that treatment of MDA-MB-231 human breast cancer cells with 2α-hydroxyursolic acid results in significant inhibition of cell metastasis, including migration, invasion and angiogenesis by downregulating MMP-2, MMP-9 activities and VEGF expression. The proposed mechanisms of action would be that 2α-hydroxyursolic acid suppressed EGF-induced EGFR phosphorylation, which inhibited EGF-induced activation of PI3K/Akt pathway, leading to translocation of NF-κB and AP-1 to nuclear, resulting in inhibition of the expression of VEGF and MMP-2 and MMP-9 activities (Figure 4.10). Based on our results, diet rich in fruits and vegetables with bioactive compounds, 2α-hydroxyursolic acid in this case, might be beneficial to prevent or coordinate with treatment of breast cancer metastasis. Signal transduction and activator of transcription (STAT) pathway, mitogen-activated protein kinase (MAPK) pathway and other signaling pathways have also been reported to involve in cancer metastasis.[5, 82]

Therefore, the mechanisms of action of 2α-hydroxyursolic acid's anti-metastasis effect need further studied. Further *in vivo* studies are necessary to verify the efficacy of this compound as a potential candidate for breast cancer prevention and treatment.

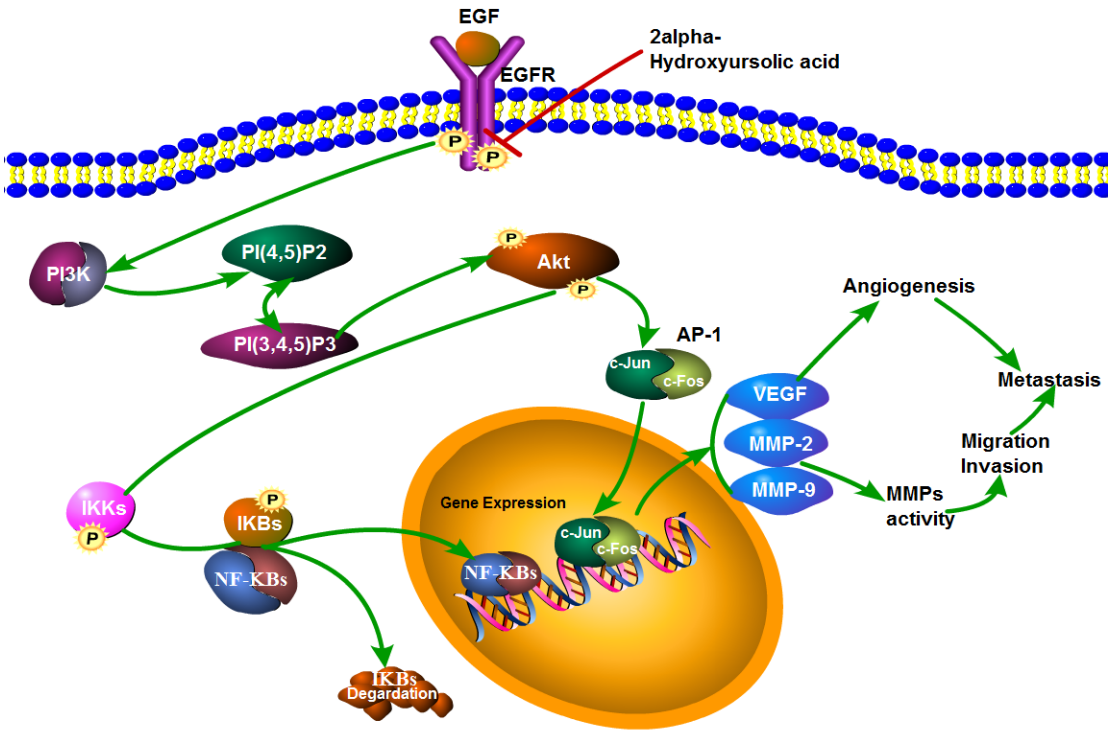


Figure 4.10. Potential mechanisms of action of 2α-hydroxyursolic acid in inhibiting cell metastasis in MDA-MB-231 human breast cancer cells through blocking EGF/EGFR-PI3K/Akt-NF-κB/AP-1 signal transduction pathway.

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