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THE POTENTIAL EFFECT OF TRITERPENOIDS IN TRIPLE NEGATIVE BREAST
CANCER

By

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ABSTRACT OF THE THESIS

The potential effect of triterpenoids in triple negative breast cancer

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Triple negative breast cancer (TNBC) classifies as lacking expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her-2). TNBC mainly represents basal-like subtype, which consists 80% of TNBC. TNBC has the poorest prognosis and the current standard therapy is chemotherapy.

Triterpenoids have been reported as chemopreventive agents for many cancers, including breast cancer. Since natural triterpenoids such as oleanolic acid and ursolic acid were reported to have weak anti-inflammation and anti-cancer effects, synthetic oleanane triterpenoids have been developed to increase biological activity. One such synthetic oleanane triterpenoid, 2-cyano-3, 12-di oxooleana-1, 9-dien-28-oic acid-Imidazolidine (CDDO-Im), is a promising agent with potent anti-proliferation and apoptosis activity against various cancers, but the mechanism of these effects remains unclear.

In the first part of the project, CDDO-Im inhibited the cell growth, induced G2/M arrest and apoptosis in triple negative breast cancer SUM159 cells. Western blot analysis showed CDDO-Im down-regulated cyclin D1, up-regulated apoptosis markers, and increased DNA damage checkpoint markers that lead to G2/M arrest. Moreover, CDDO-

Im repressed the protein level of CD44, pSTAT3 and pNFκB in a dose dependent manner, suggesting that CD44-STAT3 or NFκB signaling may play an important role for the chemopreventive action of CDDO-Im in triple negative breast cancer.

In the second part of the project, CDDO-Im decreased CD44⁺/CD24⁻ cell population in SUM159 monolayers and CD44⁺/CD24⁻/ESA⁺ cell population, which presents as cancer stem cell (CSC) population, in SUM159 mammospheres. CDDO-Im also reduced the number and size of primary and secondary mammospheres in a dose dependent manner.

In conclusion, CDDO-Im inhibited TNBC by inducing the G2/M arrest and apoptosis. CDDO-Im had anti-cancer stem cell effect by decreasing the CSC population. These findings suggest the potential use of CDDO-Im for the chemoprevention of TNBC and warrant further *in vivo* studies and clinical evaluation.

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LIST OF ABBREVIATION

AECHL-1	<i>Ailanthus excelsa</i> chloroform extract-1
Bcl2	B cell lymphoma 2
BCSC	Breast cancer stem cell
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
c-Casp	Cleaved-caspase
CDDO	2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid
CDDO-EA	CDDO-ethyl amide
CDDO-Im	CDDO-Imidazolid
CDDO-MA	CDDO-methyl amide
CDDO-Me	CDDO-methyl ester
CDK	Cyclin dependent kinase
CSC	Cancer stem cell
CYCB1	Cyclin B1
CYCD1	Cyclin D1
CYCE	Cyclin E
COX-2	Cyclooxygenase-2
c-PARP	Cleaved-Poly (ADP-ribose) polymerase
DFS	Disease-free survival
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ESA	Epithelial specific antigen
ErbB	Epidermal growth factor
HDL	High density lipoprotein
HER-2	Human epidermal growth factor receptor-2
IFN γ	Interferon γ

IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MFE	Mammosphere forming efficiency
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
OA	Oleanolic acid
ORR	Overall response rate
OS	Overall survival
p-Akt	Phospho-Akt
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
pCR	Pathologic complete response
PFS	Progression-free survival
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SO	Synthetic oleanane triterpenoid
STAT	Signal transducer and activator of transcription
TNBC	Triple negative breast cancer
TNF- α	Tumor necrosis factor α
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
UA	Ursolic acid
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

Breast cancer is the most common cancer in women between the ages of 40 and 55 [1] with more than approximately 1,300,000 new cases diagnosed resulting in 450,000 deaths each year [2]. Based on the World Health Organization (WHO) database, the incidence of breast cancer varies greatly depending on race, age and body type. Specifically, African-American women have a 6% lower incidence rate but have 17% higher breast cancer death rate than Caucasian women [3, 4]. Breast cancer is a heterogeneous disease and classifies into three basic therapeutic groups in the clinical field: estrogen receptor (ER) positive, human epidermal growth factor receptor-2 (Her-2, also called ErbB2) positive and triple negative groups [2]. Breast cancer can be further classified into four major intrinsic subtypes based on the gene expression profile: luminal A, luminal B, Her-2 positive and basal-like [2].

1.1 Subtypes of breast cancer

1.1.1 Estrogen receptor (ER) positive

Estrogen receptor positive breast cancer represents 75~80% of all breast cancer cases [5] and is divided into two subtypes: luminal A and luminal B. Luminal subtypes activate ER-responsive genes, presumably of luminal epithelial cells origin because of the similarities of the expression of estrogen receptor, and express luminal cytokeratin 8/18 [6]. Luminal A subtypes are ER or progesterone receptor (PR) positive and express high level of ER-associated genes, but are Her-2 negative. Luminal B subtypes identify as ER or PR positive and Her-2 positive [6]. Luminal A have better prognosis than luminal B because luminal B express low level of ER and high cell proliferation rate [7, 8]. Luminal

A typically responds more effectively to selective estrogen receptor modulators such as tamoxifen [5, 6, 9].

Estrogens play a key role in breast cancer, but the precise mechanism remains unclear. *Russo et al.* suggested that the mechanism may be dependent on the activation of the ER. Their results showed that estrogen and its metabolites induced cell proliferation, caused genomic changes and damage that lead to carcinogenesis [10]. Another possible mechanism is that estrogen may increase oxidative stress in cells, which plays a key role in mammary cancer development [11, 12].

The current treatment for estrogen receptor positive breast cancer is endocrine therapies [13]. Tamoxifen, an antagonist of ER, has been the first-line agent of adjuvant therapy for patients with ER positive breast cancer [14, 15]. However, some patients have *de novo* resistance to tamoxifen. One study suggested that tamoxifen might act as an estrogen agonist, which increases high levels of AIB1 (an ER coactivator) and Her-2. It might increase the cross talk between ER and Her-2 pathways in breast cancer cells [16]. Another option for treatment of ER positive breast cancer is aromatase inhibitors, which reduce the activity of cytochrome P-450 aromatase to decrease the circulating estradiol levels [17, 18]. For aromatase inhibitors such as letrozole, anastrozole and exemestane, research studies showed that these agents have high selectivity and repress 85-92% plasma estradiol levels. Although endocrine resistance may still develop in patients, non-steroidal such as toremifene, idoxifene and steroidal agents such as fulvestrant may be other options for patients [17, 18].

1.1.2 Human epidermal growth factor receptor-2 (Her-2) positive

Her-2 (also called ErbB-2) is a transmembrane receptor tyrosine kinase, a member of the epidermal growth factor family that is a potent mediator of normal cell growth and development [19]. Her-2 positive breast cancer presents the overexpression or amplification of the ErbB-2 gene and exhibits 15~20% of breast cancer cases [20]. This subtype presents Her-2 positive but ER negative with poor differentiation [21]. Her-2 positive also expresses high level of *GRB7* genes, which is located close to Her-2 in the genome [6, 8, 9].

The first-line treatment agent for Her-2 positive breast cancer patients is trastuzumab, a recombinant monoclonal antibody recognizes p185^{HER2}. Trastuzumab down-regulates Her-2 expression by accelerating receptor endocytosis and inhibits cell cycle progression by inducing the formation of p27^{Kip1}/Cdk2 complex [22, 23]. However, some clinical cases reported cancer was recurred after the adjuvant therapy with trastuzumab [24]. Another alternative agent is lapatinib (Tykerb, Glaxo-SmithKline), an orally active small molecule that inhibits the tyrosine kinases of Her-2 and epidermal growth factor receptor type 1 (EGFR), and this agent is used in the combination therapy with capecitabine [24].

1.1.3 Triple negative breast cancer

Breast cancers lack the expression of ER, PR and Her-2 are known as triple negative breast cancer (TNBC), which represents 12-15% of breast cancer cases [8, 25, 26]. Although TNBC is not the most common breast cancer in women worldwide, it is the most aggressive type of breast cancer. TNBC has the highest percentage of breast

cancer deaths [27]. In addition, patients with TNBC has high recurrence but there are limited treatments [27, 28] because TNBC is resistant to the treatments for ER positive and Her-2 positive breast cancer [25].

1.2 Subclassification of triple negative breast cancer

1.2.1 Basal-Like subtype

Basal-like subtype presents nearly 80% of TNBC [29]. It lacks the expression of ER, PR and Her-2 and has high expression of proliferation-related genes, epidermal growth factor receptor (EGFR) and specific cytokeratins such as CK5/6, CK14 and CK17 [9, 20, 30, 31]. Other identification markers are p16, p53, phospho-histone and c-Myc [7]. Basal-like subtype also presents $CD44^{+}/CD24^{-}$ phenotype, which is associated with stem-cell profile [32] and frequent mutations in *TP53* [8, 9, 33, 34].

In clinical studies, basal-like subtype has poor outcome and is associated with an aggressive profile, which develop distant metastasis (especially the first 5 years), shorter survival and high mortality rate [35]. The prevalence of basal-like breast cancer is highest among premenopausal African-American women [36]. Most of basal-like tumors show *de novo* resistance to endocrine and Her-2 targeted therapies [35]. Therefore, the conventional chemotherapies have been the only option for the patients with basal-like breast cancer.

1.2.2 Breast cancer-associated gene 1 (BRCA1)-mutation subtype

BRCA1-mutation presents 10% of TNBC [20], and 75% of BRCA1-mutation breast cancers is related to basal-like breast cancers [27]. Basal-like breast cancers

without BRCA1-mutation relate to “BRCAness” by having high tumor grade, lymphocytic infiltrate, ER negative, Her-2 negative, *TP53* mutations, or c-Myc amplification [37]. Although BRCA1-mutation is one of the main causes of TNBC, *Meyer et al.* indicated that BRCA2-mutation is also a risk factor of TNBC [38]. Even BRCA2 gene has lower mutation frequencies than BRCA1 gene to develop TNBC [38, 39]. BRCA1 and BRCA2 genes are tumor suppressors and play critical roles in protecting genome integrity through homologous recombination, but act at different stages in DNA damage response and DNA repair [39]. Patients with mutation in both BRCA1 and BRCA2 are significantly in a high risk of developing breast cancer than patients in general population [40].

1.2.3 Claudin-low subtype

A new subtype identified by gene profile studies [41], named claudin-low, consists 25-39% of TNBC [8, 9, 42]. This subtype lacks the expression of claudin proteins, which link the potential space between adjacent epithelial cells and epithelial cell adhesion molecules such as E-cadherin, EpCAM and mucin-1 [8, 42]. Claudin-low subtype presents Her-2 negative, ER negative and PR negative [42]. It has low expression of CK18/19 and inconsistent expression of CK5, CK14 and CK17 [42]. It also presents $CD44^{+}/CD24^{-/low}$ and $CD49f^{+}/EpCAM^{-/low}$ phenotype, which are enriched in breast tumor initiating cells [42-44]. Claudin-low subtype also has increased epithelial-to-mesenchymal transition, which trigger cells to be invasive and migratory with stem cell features [42, 45]. Table 1.1 summarizes the characteristics of breast cancer subtypes.

1.3 Risk factors of triple negative breast cancer

TNBC with basal-like subtype has specific expression profile such as high expression of Ki-67, laminin and p53 and low expression of Bcl-2, an anti-apoptotic marker [46]. TNBC is highly invasive and has a high incidence of lymph node involvement [20]. Clinically, they have high nuclear mitotic grade and large tumor size [46]. Taken together, these factors may be a reason for patients to have poor overall survival and high risk of early recurrence with most deaths occurring in the first 5 years of diagnosis [46]. There is also rapid progression from the onset of metastasis to death and strong correlation between obese persons and chemosensitivity [20].

TNBC develops early in life and more often in pre-menopausal women [47, 48]. Individuals with metabolic syndrome such as high blood glucose, triglyceride or low high density lipoprotein (HDL) [49], increased body weight [50] and younger age, which is identified as less than 50 years old at diagnosis [51, 52] are considered as high risk groups of TNBC. In addition, individuals who are of Hispanic or African-American ethnicity [47, 53], has never breast-fed or used oral contraceptives [36, 54] have higher chance to develop TNBC. Mutations in BRCA1 also correspond with the risk of developing basal-like breast cancer or TNBC [25].

1.4 Current therapies for triple negative breast cancer

1.4.1 Adjuvant /Neoadjuvant chemotherapies

The current standard therapy for TNBC is chemotherapy [20]. Chemotherapies divide into two options: adjuvant chemotherapy and neoadjuvant chemotherapy.

Adjuvant therapy can further divide into two groups: cytotoxic agents, which have DNA-

damaging effect to rapidly dividing cells and targeted therapies such as inhibition of poly-ADP ribose polymerase I (PARP1) and EGFR [28, 55-57].

Neoadjuvant therapy defines as having higher success in treating inoperable tumors and reduces the probability of lymph node involvement by giving agents prior to the main treatment [58]. Neoadjuvant therapies show high response rates for tumor regression and improve the rate of breast conservation [59]. Although there has been no difference in overall survival (OS) between neoadjuvant and adjuvant groups, patients treated with neoadjuvant therapies, whose tumors had higher pathologic complete response (pCR) at surgery, improved disease-free survival (DFS) and OS rates [59].

1.4.2 Targeted therapies

Potential targets for treatment include surface receptor such as epidermal growth factor receptor (EGFR), protein kinase components of the protein kinase B (Akt) pathway such as PI3K or inhibition of defective DNA repair such as PARP1 [25].

PARP is a nuclear protein that is activated in the presence of DNA damage. PARP1 and PARP2 have been related to DNA stability [60]. Single-strand DNA breaks are usually removed by base excision repair and PARP1 represents one of the central components. In the absence of PARP1, single-strand breaks build up and are converted at the replication fork to double-strand DNA breaks, which cannot be repaired in BRCA1-mutated cells [30, 60].

EGFR is a growth factor receptor tyrosine kinase, and/ or its related ligands, such as TGF- α , have been identified as a component of promoting tumor growth in multiple cancer types [61]. Sixty percents of TNBC with basal-like subtype have the

overexpression of EGFR [8, 62]. TNBC with EGFR overexpression has low response to chemotherapy and has poor overall survival [8].

Phosphatidylinositol 3-kinase (PI3K) relates to the growth and survival signaling, which is from tyrosine kinase or RAS protein to downstream proteins such as Akt and mTOR [63]. PI3K is a key protein in cell proliferation and survival that is activated in TNBC by PTEN loss or PIK3CA mutation [64, 65]. More than 60% of TNBC exhibits the loss of PTEN expression [64], which is related the downstream activation of Akt and mTOR [66, 67]. Therefore, PTEN-dependent activated signaling pathway is a potential target for the patients with TNBC.

Another potential target molecule is vascular endothelial growth factor receptor (VEGF), a key driving force behind malignant blood vessel growth [68]. VEGF is a inducer in tumor angiogenesis and relates to the tumor growth in many cancers, including breast cancer [69]. Therefore, blocking VEGF or its receptor is an ideal therapeutic target, which leads to the clinical trials of a humanized monoclonal antibody against VEGF, bevacizumab [69].

1.4.3 *In vitro* and *in vivo* studies in triple negative breast cancer

Platinum-based agents: *Bhattacharyya et al.* demonstrated BRCA1-mutated cells were more sensitive to cisplatin treatment and cisplatin would cause DNA cross-link damage in cells [70]. The chemosensitivity to cisplatin is mediated by p63/p73 regulation in BRCA1-mutated cells [71]. High dose cisplatin treatment can reduce tumor volume in BRCA1/p53 mutated tumors from mouse mammary models [72].

PARP inhibitors: *Farmer et al.* demonstrated BRCA1 or BRCA2-deficient cells were sensitive to a PARP inhibitor KU0058948 than wild type cells, which the effect was rapid and irreversible [55]. In 43 human breast cell lines, *Finn et al.* found triple negative subtypes were more sensitive to a PARP inhibitor AZD2281 than other subtypes. AZD2281 could further enhance the effect of cisplatin treatment in breast cancer cells [73]. The efficacy of platinum-based treatments such as cisplatin and carboplatin is enhanced by the combination with AZD2281 in BRCA1/p53 mutated tumors in mice [74]. In the same study, they also found AZD2281 inhibited tumor growth in mice without signs of toxicity and increased the survival rate [74].

EGFR inhibitors: *Hoadley et al.* reported basal-like subtypes were more sensitive than luminal subtypes [75]. *Oliveras-Ferraros et al.* indicated cetuximab significantly enhanced cisplatin-induced apoptotic cell death in a gefitinib resistant MDA-MB-468 breast cancer cells [76]. Also, cetuximab as a single agent was efficient in increasing BRCA1 protein levels [76]. Preclinical results supported a synergistic effect of EGFR inhibition in TNBC when utilizing chemotherapy and gefitinib, an inhibitor of EGFR tyrosine kinase domain [77].

PI3K inhibitors: *Lehmann et al.* indicated that TNBC cells such as CAL-51 and SUM159 cells were more sensitive to a PI3K inhibitor NVP-BEZ235 than basal-like cells such as HCC1806 and MDA-MB-468 cells [78].

1.4.4 Clinical studies in triple negative breast cancer

Cytotoxic agents: taxanes and anthracycline improved pathologic complete response (pCR) rates and survival rates in stage I-III TNBC patients [28, 79]. *Liedtke et*

al. also showed patients with TNBC treated with fluorouracil, doxorubicin, cyclophosphamide/ fluorouracil, epirubicin and cyclophosphamide had significant improved pCR rates (22%) compared to non-TNBC patients (11%) [79].

Platinum-based agents: Several studies evaluated agents as monotherapies such as cisplatin or combination strategies like epirubicin and cisplatin [59]. *Sirohi et al.* studied 328 patients (62 patients with TNBC) received 5-fluorouracil 200 mg/m² by daily 24-h continuous infusion for 18 weeks with epirubicin 60 mg/m² i.v. and cisplatin 60 mg/m² i.v. both repeating three weekly for six courses as a part of clinical trial [26]. The results showed that neoadjuvant complete response rates, the 5-year OS, 5-year disease-free survival and overall response rate were significantly improved in TNBC patients. Patients with TNBC also had prolonged progression-free survival of 6 months after the treatment [26]. Twenty-eight women with stage II or III TNBC were treated with four cycles of cisplatin at 75 mg/m² every 21 days. The results showed that the pCR rate was 22%, and the partial and complete clinical response was 64% and 50%, respectively in TNBC patients. The results indicated that patients have good responses with cisplatin treatment [80].

Bevacizumab (antiangiogenic agent): In E2100 study, they compared paclitaxel alone or in combination with bevacizumab in metastatic breast cancer patients. This phase III clinical trial demonstrated a significant improvement in progression-free survival (PFS) and overall response rate (ORR) with paclitaxel in combination with bevacizumab when compared with paclitaxel alone [81]. Results suggested bevacizumab could enhance paclitaxel treatment [81].

PARP inhibitors: Iniparib in combination with gemcitabine and carboplatin, platinum-based agents treatment improved the clinical benefit rate, PFS and OS in patients with TNBC in randomized phase II clinical trial [82]. Similar results also found in *O'Shaughnessy et al.* study [83].

EGFR inhibitors: In TBCRC001 study, 102 patients with TNBC were treated with cetuximab alone or in combination with carboplatin, a platinum-based agent. Patients with combination therapy had a better response rate (17%) than cetuximab alone treatment (6%), indicating that cetuximab could enhance carboplatin treatment [84].

The *in vitro*, *in vivo* and clinical studies of related therapeutic agents in triple negative breast cancer are summarized in Table 1.2 to Table 1.4, respectively.

1.5 Triterpenoids and CDDO derivatives

There is still no specific treatment for patients with TNBC and there is a critical need for developing agents with less adverse effects than current therapies. Several bioactive agents such as organosulfides, berry compounds and isothiocyanates, have been discovered in plants and human diets, which have been investigated as chemopreventive agents for various cancers [1, 85].

Chemopreventive agents classified into two groups: blocking agents that prevent initiation of carcinogenesis and suppressing agents that prevent the further progression of lesions in tumors [86]. For recent years, scientists have investigated one phytochemical group called terpenoids, which had demonstrated chemopreventive effects in breast cancer [86].

1.5.1 Discovery and structures of triterpenoids

Terpenoids, also referred to as terpenes, are formed from five-carbon isoprene units that also named isoprenoids [87]. Over 40,000 different terpenoids are isolated from plants, animals, and microbial species [88, 89]. Based on the number of building blocks, terpenoids can be classified as monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, tetraterpenes and polyterpenes [86].

Triterpenoids, one of groups of terpenoids, are metabolites of isopentenyl pyrophosphate oligomers [1]. More than 20,000 triterpenoids exist in nature [90]. Triterpenoids exist in plants such as seaweeds and in wax-like coatings of fruits and herbs [1]. Triterpenoids also synthesized in many plants by the cyclization of squalene [90] and can be further classified as cucurbitanes, dammaranes, dubosanes, ergostanes, friedelanes, lanostanes, lupanes, oleananes, squalenes, and ursanes [1, 91, 92]. The structures and sources of these compounds are summarized in Table 1.5. For those diverse natural triterpenoids, oleananes (known as oleanolic acid, OA) and ursanes (known as ursolic acid, UA) had anti-inflammatory and anti-cancer effect *in vivo*, but the effects were weak [93, 94]. To have more potent anti-inflammation and anti-cancer capability, synthetic triterpenoids were established, especially synthetic oleanane triterpenoids (SO) [95, 96].

Synthetic triterpenoids derived from oleanolic acid including 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid (CDDO), CDDO-methyl ester (CDDO-Me), CDDO-imidazolid (CDDO-Im), CDDO-methyl amide (CDDO-MA), CDDO-ethyl amide (CDDO-EA), CDDO-trifluoroethyl amide (CDDO-TFEA) and dinitrile (Di-CDDO) [90]. Structures of these compounds are shown in Fig 1.1. CDDO, CDDO-Im and CDDO-Me have shown greater anti-inflammatory effect than natural oleanolic acid *in vitro* [97-99].

Its chemical structure has electron-withdrawing substituents at the C-2 position, which lead to have more biological activity [97-99]. CDDOs have also shown strong chemopreventive activities in animal models of liver, breast and lung cancers [90, 100-104].

1.5.2 The pharmacological effect of natural and synthetic triterpenoids

Although plant-derived triterpenoids have shown anti-cancer effect *in vitro*, they are mostly used in traditional herbal medicine in many Asian countries for their anti-inflammation and protection effect in the vascular system [105]. Natural triterpenoids have also been used in medicine for its hepatoprotective and cardiogenic effects [96]. For SOs, they are mostly used for its anti-inflammatory and anti-cancer effects.

1.5.2.1 Anti-inflammatory effect

The anti-inflammatory effect is common in many triterpenoids. Different mechanism is involved in inhibiting phospholipase A₂ [106, 107], 5-lipoxygenase (5-LO) [108] and human leukocyte elastase (HLE) [96]. In addition, triterpenoids from olive oil reduced the expression of interleukin (IL)-6 and tumor necrosis factor α (TNF- α) in lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages [109]. Oleanolic acid, erythrodiol and uvaol from olive oil inhibited the production of IL-1 β and IL-6 [110].

SOs suppressed the induction of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in macrophages that stimulated by pro-inflammatory proteins

such as interferon γ (IFN γ), TNF- α , IL-1 β , LPS and inhibited the production of inflammatory cytokines such as IL-6 both *in vivo* and *in vitro* [97, 98, 111, 112].

1.5.2.2 Hepato- and cardio-protective effects

Ursolic acid (UA) and oleanolic acid (OA) have been studied for hepatoprotective effects. The research of oleanolic acid for hepatoprotective effect was first shown in 1975 (Human Medical Institute, 1975). The mechanism of effect may be multiple. It suppressed the enzymes that related to liver damage such as cytochrome P450, cytochrome b5, CYP1A and CYP2A [96, 113].

Based on the anti-oxidant [114], anti-hyperlipidemic [93, 115], anti-arrhythmic and cardiogenic effect [116] of natural triterpenoids, *Senthil et al.* investigated the cardioprotective effect of UA and OA on myocardial ischemia *in vivo* [117]. UA and OA increased activities of membrane-bound ATPase enzymes and decreased level of lipid peroxidation in heart. *Rodríguez-Rodríguez et al.* also suggested that OA is a potential vasorelaxant agent to protect the cardiovascular system [118].

1.5.2.3 Anti-cancer effect

Plant-derived triterpenoids are suggested as anti-cancer drugs because of their cytotoxicity against cancer cells and had promising results *in vitro* and *in vivo* [119]. Natural triterpenoids such as OA may act at different phase of carcinogenesis such as tumor initiation (i.e. reduce the level of ROS), promotion (i.e. apoptosis effect) and progression (i.e. inhibit angiogenesis) [120].

It has been shown that CDDO, CDDO-Me and CDDO-Im regulated cell proliferation molecules such as cyclin D1, p21, PCNA and c-Myc [121-123] and influenced NF κ B, JAK/STAT3, PTEN/PI3K/Akt and mTOR signaling pathways to inhibit proliferation [101, 124-127]. SOs induced apoptosis in leukemia, human osteosarcoma cells, myeloma cells, lung cancer and breast cancer [128-133]. *Suh et al.* indicated that SOs also induced differentiation of human leukemia cells, adipogenic differentiation of 3T3-L1 fibroblasts and neuronal differentiation of PC12 cells [98].

1.6 Chemoprevention by natural and synthetic triterpenoids in breast cancer

The cancer preventive activity of natural and synthetic triterpenoids has been investigated in numerous *in vitro* and *in vivo* studies. Results of these studies suggested that both natural and synthetic triterpenoids could serve as potent chemopreventive agents in cancers, including leukemia [134, 135], myeloma and lung cancer [133, 136], ovarian carcinoma [137], prostate cancer [138], and liver cancer [139].

1.6.1 *In vitro* effects of natural and synthetic triterpenoids in breast cancer

Natural triterpenoids demonstrated the inhibitory effects against proliferation, growth and invasion of breast cancer [1]. Cucurbitanes, isolated from the cucurbitaceae family, present a group of triterpenoids that contain cucurbitane skeleton [140]. Cucurbitacin I inhibited the proliferation of MDA-MB-468 human breast cancer cells and reduced STAT3 activation [141]. Cucurbitacin Q, analogs of cucurbitacin I, also inhibited STAT3 activation and induced apoptosis in MDA-MB-435 and MDA-MB-453 human breast cancer cells [142]. Celastrol and Pristimerin, compounds from friedelanes family,

have anti-proliferative effect in MCF7 cells [143]. Pristimerin induced apoptosis by caspase activation and released cytochrome c in breast cancer cells [144]. Betulinic acid, a pentacyclic lupane triterpene [145], induced apoptosis and reduced expression of Bcl-2 and cyclin D1 in cancers [146]. Lupeol, another pentacyclic lupane triterpene from vegetables and fruits such as white cabbage, cucumber, olive, fig and mango, inhibited tumor growth and induced apoptosis in breast cancer [147]. Remangilonones A and C, oleanane triterpenoids extracts from *Physena madagascariensis*, induced apoptosis and have cytotoxic effect against MDA-MB-435 and MDA-MB-231 breast cancer cells [148]. Oleanolic acid, an oleanane triterpenoid isolated from *Glossogyne tenuifolia*, has cytotoxic activity on MDA-MB-231 and MCF7 breast cancer cells [149]. Ursolic acid, a pentacyclic ursane triterpenoid, inhibited the proliferation in MCF7 cells and exerted early cytostatic effect at G1 phase, which followed by cell death [150].

Suh et al. demonstrated the anti-proliferative effect of CDDO in ER-positive and negative breast cancer cells [98]. CDDO induced G1/S, G2/M arrest and apoptosis in MCF7, MDA-MB-231 and MDA-MB-435 breast cancer cells and regulated levels of cyclin D1 and p21^{WAF1/CIP1} [121]. CDDO-Me, a derivative of CDDO, repressed STAT3 activation in cancer cells [101, 124]. Furthermore, CDDO-Me suppressed the proliferation of 4T1 cells [101] and inhibited IL-6 induced JAK1 activity in MDA-MB-468 cells [124]. CDDO-Im, another derivative of CDDO, induced intracellular reactive oxygen species (ROS), apoptosis and G2/M arrest in BRCA1-mutated breast cancer cells [102]. The *in vitro* studies of natural and synthetic triterpenoids in breast cancer are summarized in Table 1.6.

1.6.2 *In vivo* effects of natural and synthetic triterpenoids in breast cancer

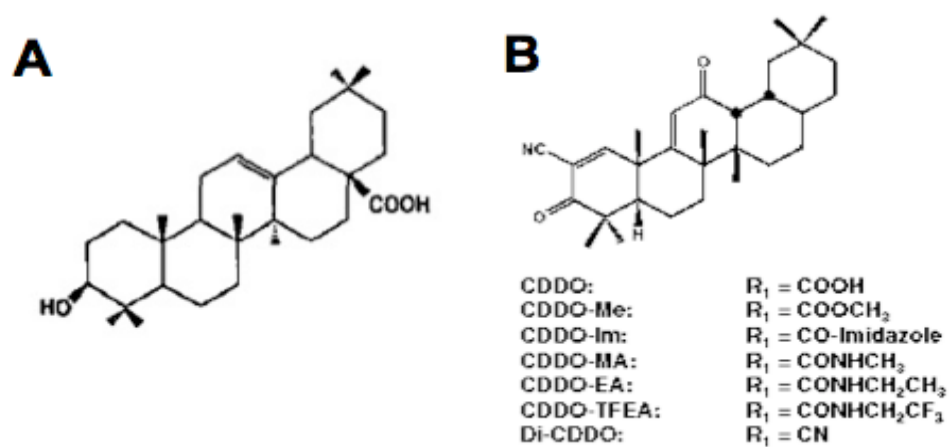
Since *in vitro* studies with both natural and synthetic triterpenoids showed potent anti-proliferative and apoptotic effect in breast cancer cells, several *in vivo* studies have been carried out to find out whether these compounds would have similar effect. To evaluate the efficiency of cucurbitacin I, *Blaskovich et al.* injected murine B16-F10 melanoma cells in nude mice and followed with cucurbitacin I treatment after 16 days [141]. Cucurbitacin I significantly inhibited the tumor growth and prolonged the survival time in mice [141]. Cucurbitacin B, an analog of cucurbitacin, reduced the MDA-MB-231 tumor growth in nude mice and decreased serum glucose level [151]. AECHL-1, a triterpenoid isolated from the root bark of *Ailanthus excelsa* Roxb, suppressed MCF7 xenograft tumor volume in nude mice [152]. Ursolic acid decreased the tumor size and growth, tumor cell proliferation and tumor interstitial fluid pressure *in vivo* [153, 154]. However, *Singletary et al.* reported that ursolic acid, which extracts from rosemary, has no effect on tumorigenesis in rats [155].

CDDO exhibited anti-tumor effect as determined by reduced tumor growth in immunodeficient mice injected with MDA-MB-435 breast cancer cells [121]. CDDO also reduced tumor growth and size of Her-2/MCF7 cells in a xenograft model of breast cancer [123]. CDDO-Im suppressed the tumor growth in MDA-MB-468 xenograft model and inhibited the tumor cell proliferation effectively by combining treatment with TRAIL, which is a ligand inducing apoptosis in cancer cells [156]. CDDO-Me repressed the mammary tumor development in BRCA1-mutant mice [157] and suppresses 4T1 breast cancer cell growth and metastasis in BALB/c mice [101]. The *in vivo* studies of natural and synthetic triterpenoids in breast cancer are summarized in Table 1.7.

1.7 Summary

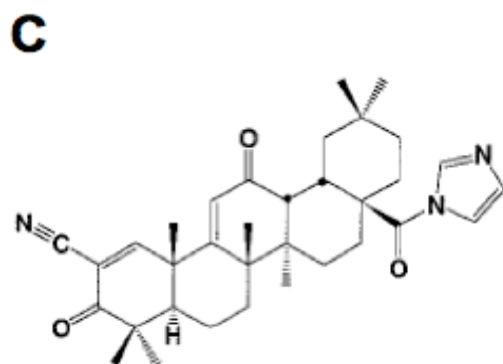
Triple negative breast cancer lacks the expression of ER, PR and Her-2. The main molecular subtype is basal-like, which consist of nearly 80% of TNBC, followed by Claudin-low subtype and BRCA1-mutation subtype. TNBC has the poorest prognosis, worse survival rate, resistant to the treatment for Her-2 positive and ER positive breast cancer, but has high chemosensitivity. For current understanding, the proper treatment for TNBC still remains limited.

Triterpenoids have been developed as potential agents for the prevention and treatment of cancer. Synthetic triterpenoids have more potent anti-tumorigenic effect than natural ones. Although the anti-cancer effects of these triterpenoids have been investigated among different cancers for several years, the understanding of its role in inhibiting TNBC remains limited and the mechanism is unclear. Therefore, further investigation of triterpenoids in TNBC is warranted.



Oleanolic acid

Synthetic oleanane triterpenoids



CDDO-Imidazole

Figure 1.1 Chemical structures of triterpenoids and their derivatives. A. Oleanolic acid B. Structures of synthetic oleanane triterpenoids and their derivatives C. CDDO-Imidazole (CDDO-Im) [104, 112, 113]

Table 1.1 Overview for breast cancer subtypes

	ER (+)		Her-2 (+)	Triple negative		
Incidence rate	75~80%		15~20%	12~15%		
Subgroups	Luminal A	Luminal B	N/A	Basal-like (80%)	BRCA-1 mutation (10%)	Claudin-low (25~39%)
ER	+	+	-	-	-	-
Her-2	-	+	+	-	-	-
Histologic grade	Variable		Variable	Mostly grade 3		
Prognosis in 5 years	Favorable	Relatively favorable	Generally adverse	Generally adverse	Generally adverse	Intermediate
Therapies	Endocrine therapy		Her-2 targeted therapy/Chemotherapy	Chemotherapy	Chemotherapy	Chemotherapy

ER: estrogen receptor; Her-2: Human epidermal growth factor receptor-2; BRCA-1: Breast cancer-associated gene 1. Table 1.1 was modified from references [62, 158, 159]

Table 1.2 *In vitro* studies of triple negative breast cancer

Cell line models	Compounds tested	Conclusions	Reference
BRCA1 ^{+/+} (E14Tg2a) and BRCA1 ^{-/-} isogenic mouse ES cell lines	Cisplatin (Platinum-based)	<ul style="list-style-type: none"> BRCA1^{-/-} mutated cells are more sensitive to cisplatin than wild type cells ↑DNA cross-link damage 	[70]
MCF-7, HCC-1937, MDA-MB-468 and T47D cells	Cisplatin (Platinum-based)	<ul style="list-style-type: none"> p63/p73 regulated pathway in cells mediates the chemosensitivity to cisplatin 	[71]
Wild-type ES cells and ES cells lacking wild-type BRCA1 or BRCA2	KU0058948 (PARP inhibitor)	<ul style="list-style-type: none"> ES cells lacking wild-type BRCA1 or BRCA2 are more sensitive to KU0058948 	[55]
Forty-three human cell lines present ER ⁺ , Her-2 ⁺ or triple negative subtypes	AZD2281 (PARP inhibitor)	<ul style="list-style-type: none"> Enhance the effect of cisplatin treatment Cells present triple negative subtypes are more sensitive to AZD2281 	[73]
MDA-MB-468 breast cancer cells	Cetuximab (EGFR inhibitor)	<ul style="list-style-type: none"> Enhance cisplatin-induced apoptotic cell death 	[76]
HCC1806 and MDA-MB-468 cells (Basal-like); CAL-51 and SUM159 cells (TNBC)	NVP-BEZ235 (PI3K inhibitor)	<ul style="list-style-type: none"> Mesenchymal-like subtypes of TNBC cells were more sensitive to NVP-BEZ235 than basal-like cells 	[78]

ES: embryonic stem; BRCA-1: breast cancer-associated gene 1; TNBC: triple negative breast cancer; PARP: poly-ADP ribose polymerase; EGFR: epidermal growth factor receptor; PI3K: Phosphatidylinositol 3-kinase

Table 1.3 *In vivo* studies of triple negative breast cancer

Animal Models	Compounds tested	Conclusions	Reference
BRCA1/p53 mutated tumors from mouse mammary model	Cisplatin (Platinum-based)	<ul style="list-style-type: none"> • ↓ Tumor volume in high dose treatment 	[72]
BRCA1 ^{-/-} ;p53 ^{-/-} mammary tumors generated in <i>K14cre;BRCA1^{F/F};p53^{F/F}</i> mice	AZD2281 (PARP inhibitor)	<ul style="list-style-type: none"> • Enhance the effect of cisplatin and carboplatin treatment 	[74]

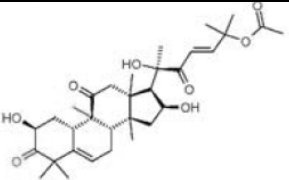
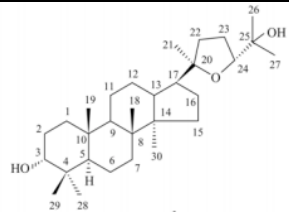
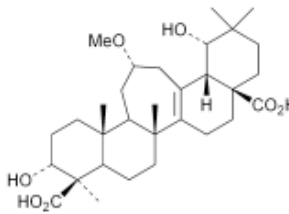
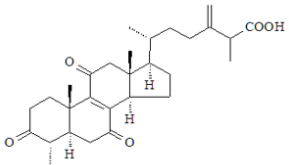
BRCA-1: breast cancer-associated gene 1; PARP: poly-ADP ribose polymerase

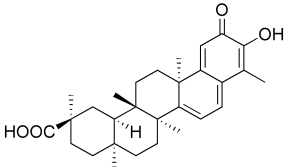
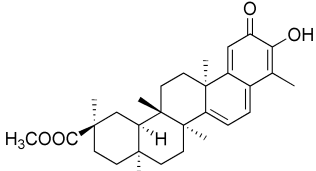
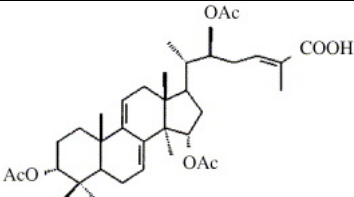
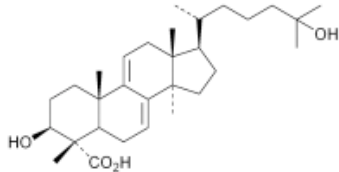
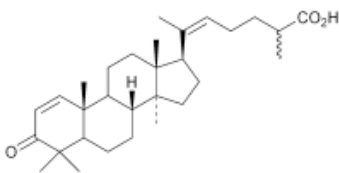
Table 1.4 Clinical studies of triple negative breast cancer

Clinical trials and participants	Compounds tested	Conclusions	Reference
Stage I-III breast cancer patients	Taxanes and anthracycline (cytotoxic agents)	<ul style="list-style-type: none"> • ↑pCR, ↑survival rates 	[79]
Three hundred and twenty-eight patients (62 patients with TNBC)	5-fluorouracil, epirubicin and cisplatin (platinum-based agents)	<ul style="list-style-type: none"> • Patients with TNBC has better complete response rates, the 5-year OS, 5-year DFS and OS than non-TNBC patients 	[26]
Twenty-eight women with stage II or III TNBC	Cisplatin (platinum-based agents)	<ul style="list-style-type: none"> • Single-agent cisplatin induced response in a subset of patients with TNBC • Decreased BRCA1 expression may identify subsets of TNBCs that are cisplatin sensitive 	[80]
Metastatic breast cancer patients (phase III clinical trial)	Bevacizumab (antiangiogenic agent)	<ul style="list-style-type: none"> • Enhanced paclitaxel treatment 	[81]
TNBC patients (phase II clinical trial)	Iniparib (PARP inhibitor) (Combine gemcitabine and carboplatin-platinum-based agents)	<ul style="list-style-type: none"> • ↑ PFS and OS 	[82, 83]
One hundred and two patients with TNBC (phase II clinical trial)	Cetuximab (EGFR inhibitor)	<ul style="list-style-type: none"> • Enhanced carboplatin treatment 	[84]

TNBC: triple negative breast cancer; pCR: pathologic complete response; OS: overall survival; DFS: disease free survival; PFS: progression-free survival; ORR: overall response rate

Table 1.5 The structures and sources of natural triterpenoids

Triterpenoids	Compounds	Structure	Sources
Cucurbitane	Cucurbitacin B		Cucurbitaceae plants [151]
Dammarane	Cabraleadiol		Leaves and wood of the tropical plant <i>Chisocheton penduliflorus</i> [160]
Dubosane	Duboscic acid		<i>Duboscia macrocarpa</i> [160]
Ergostane	Zhankuic acid A		The fruiting bodies of <i>Antrodia camphorata</i> [1]

Friedelane	Celastrol		The root bark of the Chinese medicine “Thunder of God Vine” (<i>Tripterygium wilfordii</i> Hook F.) [161]
	Pristimerin		<i>Reissantia buchananii</i> (Celastraceae) [144]
Lanostane	Ganoderic acid		Mushroom <i>Ganoderma lucidum</i> [160]
	Sexangulic acid		Chinese mangrove <i>Bruguiera sexangula</i> [95]
	Pisticialanstenenic acid		Galls of <i>Pistacia integerrima</i> [95]

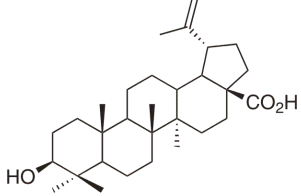
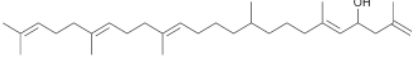
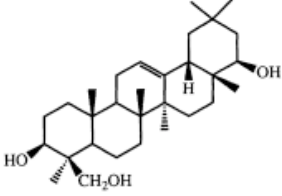
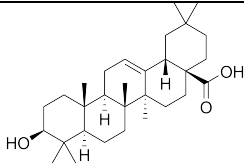
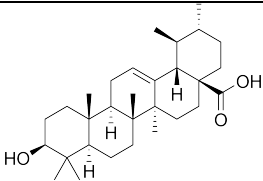
Lupane	Betulinic acid		The bark of several species of plants, principally the white birch (<i>Betula pubescens</i>) [162]
Squalene	Lobophytene		Vietnamese soft coral <i>Lobophytum</i> sp [95]
Oleanane	Soyasapogenol B (Nature as saponins)		Soybeans [160]
	Oleanolic acid		Olives, figs, rosemary [86]
Ursane	Ursolic acid		Apple peels, cranberries [163]

Table 1.6 *In vitro* studies of natural and synthetic triterpenoids in breast cancer

Triterpenoids	Compounds	Effects	Reference
Cucurbitane	Cucurbitacin I	<ul style="list-style-type: none"> • Inhibited the proliferation of MDA-MB-468 cells • ↑Apoptosis ↓ pSTAT3 	[141]
	Cucurbitacin Q	<ul style="list-style-type: none"> • Induced apoptosis in MDA-MB-435 and MDA-MB-453 cells • ↓ STAT3 activation 	[142]
Friedelane	Celastrol+ Pristimerin	<ul style="list-style-type: none"> • Anti-proliferation effect in MCF7 cells 	[143]
	Pristimerin	<ul style="list-style-type: none"> • ↑Apoptosis • Caspase activation and release cytochrome c 	[144]
Lupane	Betulinic acid	<ul style="list-style-type: none"> • ↑Apoptosis • ↓ Bcl-2, ↓ cyclin D1 	[146]
	Lupeol	<ul style="list-style-type: none"> • ↓Tumor growth • ↑ Apoptosis 	[147]
Oleanane	Remangilonones A and C	<ul style="list-style-type: none"> • Cytotoxic against MDA-MB-435 and MDA-MB-231 cells • ↑Apoptosis 	[146]
	Oleanolic acid	<ul style="list-style-type: none"> • Cytotoxic activity in MDA-MB-231 and MCF7 cells 	[149]
Ursane	Ursolic acid	<ul style="list-style-type: none"> • Inhibit the proliferation in MCF7 cells • ↑ Early cytostatic effect at G1 phase 	[150]
SO	CDDO	<ul style="list-style-type: none"> • Anti-proliferative effect in ER-positive and negative breast cancer cells 	[98]
	CDDO	<ul style="list-style-type: none"> • Induced G1/S, G2/M arrest and apoptosis in MCF7, MDA-MB-231 and MDA-MB-435 cells • Regulation of cyclin D1 and p21^{WAF1/CIP1} 	[121]
	CDDO-Me	<ul style="list-style-type: none"> • Suppressed the proliferation in 4T1 cells • ↓STAT3 activation 	[101, 124]
	CDDO-Me	<ul style="list-style-type: none"> • Inhibited IL-6 induced JAK1 activity in MDA-MB-468 cells 	[121]
	CDDO-Im	<ul style="list-style-type: none"> • ↑Apoptosis and G2/M arrest in BRCA1-mutated cells • ↑ Intracellular ROS 	[102]

SO: synthetic oleanane triterpenoids; CDDO: 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid; CDDO-Im: CDDO-Imidazolidine; CDDO-Me: CDDO-methyl ester; pSTAT3: phosphorylated signal transducer and activator of transcription 3; Bcl-2: B cell lymphoma 2; IL-6: interleukin-6; JAK1: Janus kinase 1; ROS: reactive oxygen specie

Table 1.7 *In vivo* studies of natural and synthetic triterpenoids in breast cancer

Triterpenoids	Compounds	Effects	Reference
Cucurbitane	Cucurbitacin I	<ul style="list-style-type: none"> • ↓ Tumor growth • ↑ survival time in nude mice injected with B16-F10 cells 	[141]
	Cucurbitacin B	<ul style="list-style-type: none"> • ↓ Tumor growth in nude mice injected with MDA-MB-231 cells • ↓ serum glucose level 	[151]
Ursane	Ursolic acid	<ul style="list-style-type: none"> • ↓ Tumor size and tumor cell proliferation in mice 	[153]
	Ursolic acid	<ul style="list-style-type: none"> • ↓ Tumor interstitial fluid pressure • ↓ Tumor growth 	[154]
	Ursolic acid	<ul style="list-style-type: none"> • No effect on tumorigenesis in rats 	[155]
AECHL-1	-	<ul style="list-style-type: none"> • ↓ Tumor volume in nude mice injected with MCF7 cells 	[152]
SO	CDDO	<ul style="list-style-type: none"> • Anti-tumor effect in immunodeficient mice • ↓ tumor growth in mice that injected with MDA-MB-435 cells 	[121]
	CDDO	<ul style="list-style-type: none"> • ↓ Tumor growth and size of Her-2/MCF7 cells in a xenograft model 	[123]
	CDDO-Im (Combine with TRAIL)	<ul style="list-style-type: none"> • ↓ Tumor growth in MDA-MB-468 xenograft model • ↓ Tumor cell proliferation 	[156]
	CDDO-Me	<ul style="list-style-type: none"> • ↓ Mammary tumor development in BRCA1-mutant mice 	[157]
	CDDO-Me	<ul style="list-style-type: none"> • Suppressed 4T1 breast cancer cell growth and metastases in BALB/c mice 	[101]

AECHL-1: *Ailanthus excelsa* chloroform extract-1; SO: synthetic oleanane triterpenoids; CDDO: 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid; CDDO-Im: CDDO-Imidazolide; CDDO-Me: CDDO-methyl ester; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; BRCA-1: Breast cancer-associated gene 1

Chapter 2: CDDO-Imidazolidine (CDDO-Im) inhibits the growth of triple negative breast cancer cells

2.1 Introduction

Human breast cancer is a heterogeneous disease and can be classified into different subtypes based on prognostic and therapeutic implication [164]. Triple negative breast cancer represents one of the subtypes of breast cancer, which associated with an aggressive clinical course and its clinical therapies are limited. To find out potential target treatments, chemopreventive agents were investigated *in vitro* and *in vivo* studies.

Breast cancer cell lines were proved useful for *in vitro* models and for preclinical studies [164]. *Grigoriadis et al.* classified breast cancer cell lines into three groups. The first group includes ER⁺/Her-2⁻ and ER⁻/Her-2⁺ cell lines. The second group is composed of ER⁻/Her-2⁻ basal cancer cell lines. The third group is consisted of triple negative cell lines with either having amplification or higher abundance of Her-2 and EGFR gene expression [165]. In this project, we used SUM159 cell line as *in vitro* model. This cell line classifies as ER⁻/Her-2⁻ group and also present as basal “B” breast cancer group [165], which expresses stem-cell like profile and may reflect triple negative tumor type in clinical [166]. SUM159 cells express CD44⁺/CD24⁻ phenotype [167] with wild type BRCA1 mutation [168] and the mutation in *PIK3CA*, *TP53* and *HRAS* genes [169].

Synthetic triterpenoids are known as potent anti-proliferative and pro-apoptotic agents [90] and can induce differentiation in different cell types [98], which considered as promising anti-cancer drugs. CDDO- Imidazolidine (CDDO-Im) is considered as chemopreventive agents in recent years. For previous research, CDDO-Im had anti-

cancer effect in different cancers such as lung cancer [136], pancreatic cancer [170], colon cancer [122], leukemia [134, 135] and breast cancer [86, 98, 102, 121]. Therefore, in this chapter, we proposed to determine whether CDDO-Im has the ability of inhibiting cell growth in TNBC.

We investigated the effect of CDDO-Im on cell cycle and apoptosis in TNBC. Possible mechanism was examined with related protein markers, especially cell cycle checkpoints such as Chk1, Chk2, cdc2, cyclin B1 (CYCB1) and p53. The tumor suppressor p53 regulates DNA damage cell cycle arrest [171, 172] by stimulating p21^{WAF1/CIP1}, an inhibitor of cyclin dependent kinase (CDKs) that would induces G2/M arrest by inhibiting cyclin B1/cdc2 (CDK1) [173]. Chk1 and Chk2 are downstream molecules of ATM and ATR, and they are critical for determining cellular response to DNA damage [174]. DNA damage activates Chk1 that phosphorylates and inactivates cdc25, allowing inactivation of cyclin B1/cdc2 complex, which cause G2/M arrest [175]. The activation of Chk2 would cause the effect on cdc25 and p53, which is similar to those mediated by Chk1 [174]. Other markers we would examine is cyclin E and CDK2 for G1/S phase, where cyclin E associates and activates with CDK2 [176]. With DNA damage, it activates p21^{WAF1/CIP1}, which would inhibit the activation of cyclin E/CDK2 and may cause G1/S arrest [174, 176].

2.2 Material and Methods

2.2.1 Reagents

1-[2-Cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oyl]-imidazole (CDDO-Im) was provided by Dr. Michael Sporn at Dartmouth Medical School (Hanover, NH) and dissolved in dimethyl sulfoxide (DMSO). Stock solution was prepared as 20 mM and store at -80°C freezer. For *in vitro* studies, stock solution was diluted with 1:1000 ratios to 20 µM for treatment preparation.

2.2.2 Cell culture

SUM159 breast cancer cells were provided by Dr. Li Cai at Rutgers University. Cells were maintained in Ham's F12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, 1% HEPES solution, 1.0 µg/ml hydrocortisone and 5.0 µg/ml insulin at 37°C in a humidified incubator with 5% CO₂.

2.2.3 Growth inhibition assay-MTT assay

SUM159 cells were plated into each well of 96-well plate (1,000 cells/well). On the next day the cells were treated with (1, 10, 25, 50, 75, 100 or 200 nM) or without CDDO-Im for given incubation time. At each time point, 10 µl of MTT-I solution (thiazolyl blue tetrazolium bromide, M2128, Sigma-Aldrich, St. Louis, MO) were added into each well and incubated for 5 hours, followed by addition of 100 µl of MTT-II solution (distilled water with 10% SDS and 0.01 M HCl). The plate was then incubated overnight, and the absorbance was measured with a spectrophotometer (Tecan US, Durham NC) at 560 nm with the standard absorbance at 750 nm.

2.2.4 Cell cycle analysis

SUM159 cells were incubated with (10, 50, 100, 150 or 200 nM) or without CDDO-Im for 24 hours and 48 hours. The old medium was collected and centrifuged for 3 minutes at 1200 rpm to make sure the cells in old medium were analyzed. Cells were washed with phosphate buffered saline (PBS) and trypsinized into single cell suspension. After trypsinization, cells were fixed in 70% ice-cold ethanol for 30 minutes on ice. The cells were washed twice with PBS, and then incubated for at least 30 minutes under dark condition at room temperature in 0.5 mL of PBS containing 5 µl propidium iodide (PI) solution and 50 µl RNase A solution. Cells were analyzed on a Beckman coulter gallios flow cytometer (Beckman coulter Inc., CA).

2.2.5 Apoptosis assay

SUM159 cells were incubated with (10, 50, 100, 150 or 200 nM) or without CDDO-Im for 24 hours. The old medium was collected and centrifuged for 3 minutes at 1200 rpm to make sure the cells in old medium were analyzed. Cells were washed with cold phosphate buffered saline (PBS) and incubated with accutase for single cell suspension. Cells were resuspended with Annexin V binding buffer, counted, and centrifuged 5 minutes at 1200 rpm. Cells (1,000,000 cells/ mL) were incubated with 5 µl Annexin V and 10 µl propidium iodide (PI) (FITC Annexin V Apoptosis Detection Kit II, BD Pharmingen, San Jose, CA) for 15 minutes under dark condition at room temperature. Cells were added 400 µl Annexin V binding buffer and then analyzed on a Beckman coulter gallios flow cytometer (Beckman coulter Inc., CA).

2.2.6 Western blot analysis

The protein samples were separated on 4-15% SDS-PAGE gels (Biorad, Hercules, CA) followed by transfer to a polyvinylidene fluoride membrane (PALL, Ann Arbor, MI). The membranes were blocked with 5% milk in Tris buffer for 1 hour and then incubated with the appropriate primary antibody solutions overnight at 4°C. The membranes were washed with Tris buffer and incubated with horseradish peroxidase conjugated secondary antibody solutions for 1 hour at room temperature. The protein bands were visualized using a chemiluminescence-based kit from Amersham Biosciences (Buckinghamshire, UK). The primary antibodies, CD44, which recognizes all CD44 splicing variants, cyclin D1, Bcl2, NFκB and Chk1 were from Santa Cruz Biotechnology (Santa Cruz, CA); pErk, Erk, pAkt, Akt, pSTAT3, STAT3, pNFκB, cPARP, c-Casp8, pChk1, pChk2, pcdc2, cdc2, CDK2, cyclin B1, cyclin E, pp53, p53 were from Cell signaling Technology (Danvers, MA); β-actin was from sigma (St. Louis, MO); and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2.7 Small interfering RNA analysis

SUM159 cells were plated into each well of 6-well plate (150,000 cells/well), on the next day the cells were incubated without siRNA (use Lipofectamine 2000 agent as control) or with 40 pmol of non-targeting siRNA or Chk1 siRNA targeting sequences in the Chk1 gene for 24 hours in Opti-MEM[®] I Reduced Serum Medium (Invitrogen, Life technologies, NY). The cells were incubated with (50, 100 or 200 nM) or without CDDO-Im for 24 hours in cell culture medium. Chk1 siRNA and non-targeting siRNA

were from Santa Cruz Biotechnology (Santa Cruz, CA) and Lipofectamine 2000, the transfecting agent, was from Invitrogen (Life technologies, NY). The protocol was followed with the manufacturer's procedure (Invitrogen).

2.2.8 *In vitro* experiment design

SUM159 cells were provided by Dr. Li Cai as described above. Cells were taken from the nitrogen tank and passed. After at least 10 passages in Ham's F12 medium, a stable cell set, which checked with cell cycle analysis, was obtained and then used for *in vitro* studies. MTT assay was first used to examine the growth inhibitory effect of CDDO-Im in SUM159 cells. The appropriate time point and dose treatment of CDDO-Im for further experiment were determined. The effect of CDDO-Im in SUM159 cells was determined by cell cycle analysis and apoptosis assay. Western blot and siRNA analysis would follow the similar setting as cell cycle analysis and apoptosis assay with the appropriate markers for cell proliferation, apoptosis and cell cycle checkpoint to confirm the results from those assays and find out the possible mechanism of action by CDDO-Im in SUM159 cells.

2.2.9 Statistical analysis

Statistical significance was evaluated using the Student's *t* test or one-way analysis of variance (ANOVA).

2.3 Results

2.3.1 CDDO-Im inhibits cell growth in SUM159 cells

To determine the inhibitory effect of synthetic triterpenoids on cell growth in triple negative breast cancer cells, SUM159 cells were treated with CDDO-Im for up to 72 hours and analyzed by MTT assay. CDDO-Im was significantly inhibited cell growth in SUM159 cells (Fig. 2.1). There was no significant difference between the control group and low dose groups (Fig. 2.1A: 1 and 10 nM; Fig. 2.1B: 10 and 25 nM). CDDO-Im reduced cell growth in a dose dependent manner (Fig 2.1). In Fig. 2.1A, 50 nM treatment had significant inhibitory effect and high dose treatment such as 100 nM and 200 nM had more significant inhibitory effect in SUM159 cells. There was no difference between 100 nM and 200 nM groups (Fig 2.1A). To identify the dose range of the inhibitory effect of CDDO-Im, low dose range was tested. In Fig. 2.1B, CDDO-Im reduced cell growth in a dose dependent manner and 50 nM had shown significant inhibitory effect. There was no difference between 75 nM and 100 nM groups. Collectively, SUM159 cells had no significant response in low dose treatment that below 50 nM. Above 50 nM, CDDO-Im had significant growth inhibitory effect in SUM159 cells.

2.3.2 CDDO-Im induces cell cycle G2/M arrest in SUM159 cells

To determine further inhibitory effect of CDDO-Im in SUM159 cells, we tested the cell cycle distribution in SUM159 cells by flow cytometry. SUM159 cells were treated with 10, 50, 100, 150 or 200 nM CDDO-Im for 24 hours and 48 hours. There were no significant changes in low dose treatment such as 10 and 50 nM in 24 and 48 hours (Fig 2.2, Fig 2.3). In 100 nM treatments, there had no significant changes in cell population in

24 hours (Fig 2.2), but the cell population was slightly changed in 48 hours (Fig 2.3). In high dose treatment such as 150 and 200 nM, cell population had significantly G2/M arrest and G0/G1 phase was slightly decreased in 24 and 48 hours (Fig 2.2, Fig 2.3). Collectively, CDDO-Im induces G2/M arrest in SUM159 cells in a dose dependent manner.

2.3.3 CDDO-Im induces late apoptosis in SUM159 cells

Based on the previous results, CDDO-Im has the inhibitory effect on cell growth in SUM159 cells. We want to further clarify whether CDDO-Im would induce apoptosis in SUM159 cells that cause the inhibition on cell growth. SUM159 cells were treated with 10, 50, 100, 150 or 200 nM CDDO-Im for 24 hours and stained with FITC-Annexin V, a biochemical marker for early apoptosis. According to the manufacturer's protocol, early apoptosis stands for Annexin V⁺/PI⁻, whereas cells in late apoptosis or already dead cells stands for Annexin V⁺/PI⁺. Annexin V⁺/PI⁻ area was increased in 10, 50 and 100 nM CDDO-Im treatment in a dose dependent manner (Fig 2.4). In high dose treatment such as 150 and 200 nM, Annexin V⁺/PI⁺ and Annexin V⁻/PI⁺ were increased in SUM159 cells (Fig 2.4). Collectively, CDDO-Im induces early apoptosis in SUM159 cells within low dose treatment and induces late apoptosis in high dose treatment. Also, 100 nM CDDO-Im might be an effective dose for the treatment *in vitro*.

2.3.4 CDDO-Im inhibits cell proliferation and induces apoptosis and G2/M arrest in SUM159 cells

According to the results from cell cycle analysis and apoptosis assay, we determined protein markers of proliferation, apoptosis and cell cycle checkpoint to find out the possible mechanism for growth inhibitory effect of CDDO-Im in SUM159 cells. SUM159 cells were treated with CDDO-Im (50, 100 or 200 nM) for 24 hours and samples were analyzed by Western blot analysis. Cyclin D1, a marker for cell proliferation, was down-regulated by CDDO-Im (Fig 2.5A). cPARP and c-Casp8, markers for apoptosis, were up-regulated and Bcl2, a marker for early apoptosis, was down-regulated by CDDO-Im (Fig 2.5A).

To further examine the involvement of molecules in G2/M phase, we investigated proteins such as pChk1, pChk2, pcdc2, cyclin B1, pp53 and p53. pChk1 and pChk2 was up-regulated and Chk1 was down-regulated in a dose dependent manner (Fig 2.5B). There were no significant changes of the protein levels of pcdc2, cdc2 and p53 (Fig 2.5B). pp53 was up-regulated and cyclin B1 was down-regulated in a dose dependent manner (Fig 2.5B). We also examined the protein levels of CDK2 and cyclin E, markers for G1/S phase, and there were no significant changes with the treatment (Fig 2.5B). These results indicated that CDDO-Im activated Chk1 and Chk2 kinases and blocked the G2/M phase transition, which caused G2/M arrest. Fig 2.6 shows a schematic diagram of proposed mechanism of action of CDDO-Im on cell cycle and G2/M arrest in TNBC.

2.3.5 Chk1 might not be the key regulator in G2/M arrest by CDDO-Im

Chk1 is a checkpoint kinase responding to cellular signals to DNA damage, which would initiate cell cycle arrest and lead to DNA repair and cell survival [174]. In Fig 2.5B, we found the up-regulation of pChk1 and pChk2 and down-regulation of Chk1. Therefore, we assumed Chk1 may be a key protein that causes CDDO-Im to inhibit the cell growth and induce G2/M arrest in SUM159 cells. SUM159 cells were transfected with non-target and Chk1 siRNA and were then treated with CDDO-Im (50, 100 or 200 nM) for 24 hours and analyzed by Western blot analysis. Chk1 protein was significantly down-regulated by Chk1 siRNA, but pChk1 was still up-regulated by the treatment with CDDO-Im (Fig 2.7). These results suggest that Chk1 might not be the key regulator in G2/M cell cycle arrest mechanism by CDDO-Im.

2.3.6 CDDO-Im decreases the protein level of CD44, pSTAT3 and pNFκB in

SUM159 cells

To identify downstream signaling pathway that may be influenced by CDDO-Im, the protein levels of CD44 and the potential downstream signaling markers such as pAkt, pErk, pSTAT3 and pNFκB were measured. CDDO-Im down-regulated the protein levels of CD44, pSTAT3 and pNFκB in a dose dependent manner, whereas the protein level of pErk was up-regulated and pAkt was not changed (Fig 2.7). Total protein levels of STAT3, Akt, Erk and NFκB were not influenced by the treatment with CDDO-Im (Fig 2.7). These results indicated that CDDO-Im might affect CD44-STAT3 signaling pathway or pNFκB pathway in SUM159 cells.

2.4 Discussion

Our results clearly showed that CDDO-Im inhibits the cell growth and induces apoptosis and G2/M arrest in TNBC. With respect to dose dependent experiments, concentration between 100 nM and 200 nM seems optimal. In MTT assay, treatment with 50 nM CDDO-Im showed significant inhibitory effect in TNBC. However, in cell cycle analysis and apoptosis assay, treatment with low dose of CDDO-Im such as 10 and 50 nM did not show significant changes, while treatment with over 100 nM CDDO-Im had significant results. The result with the treatment with 50 nM CDDO-Im did not correlate with MTT assay where significant effects were seen. The reason might be due to the difference of cell density with the treatment. The cell density in MTT assay was lower than the cell density in cell cycle analysis and apoptosis assay, which means cells in MTT assay expose more CDDO-Im than cells in cell cycle analysis and apoptosis assay. This result may influence the sensitivity to the treatment in the cells. For future experiments, the cell density with the treatment should correlate with each assay to maintain the same condition. Treatment with 200 nM CDDO-Im induces significant G2/M arrest and late apoptosis, which may be toxic to SUM159 cells. Compared to the dose usage of CDDO-Im (300 nM to 1 μ M) from other studies [102, 136, 137], the dose 200 nM we used in this project was relatively low, suggesting that SUM159 cells may be sensitive to the treatment with CDDO-Im.

CDDO-Im was first shown to inhibit DNA synthesis in 2003 [112]. *Kim et al.* first showed CDDO-Im induced ROS accumulation, DNA damage and activated cell cycle checkpoint signaling in BRCA1 mutated breast cancer cells [102], which is similar to our results in SUM159 cells. When DNA is damaged, G2/M checkpoint prevents cells

entering mitosis to maintain genomic stability and reduce cancer risk [177]. Chk1 is a key checkpoint kinase that is responsible for cellular signals to DNA damage, which would initiate cell cycle arrest [174]. We found that the levels of pChk1 and pChk2 were up-regulated by CDDO-Im, which may target the Chk1/Chk2/cdc2/cyclin B1 pathway as seen in Fig 2.5B and 2.6. However, transfection with Chk1 siRNA revealed that Chk1 is not essential for G2/M arrest by CDDO-Im in SUM159 cells (Fig 2.7). These results suggested that Chk1 might not be a major molecule in the G2/M arrest. For future research, other knockdown molecule is needed to examine to find out the major regulator such as Chk2, another upstream molecular in Chk1/Chk2/cdc2/cyclin B1 pathway. We found that CDDO-Im increased the level of pChk2 and its downstream molecules in SUM159 cells.

CD44 and its associated proteins are key molecules that regulate extracellular matrix proteins involving in cell growth, survival and differentiation [178]. The overexpression of CD44 is related to breast cancer progression as well as metastasis, which consider as an indicator of poor prognosis [179, 180]. CD44 is a ligand-binding surface protein, which will regulate intracellular signaling and cellular response by interacting with extracellular molecules such as receptor tyrosine kinases or intracellular kinases [178, 179]. With different types of cancers, the target signaling molecules and the downstream pathway related to CD44 may be different. Therefore, identification of targeting molecules is a way to find out possible treatment for TNBC.

In our study, we found that CDDO-Im down-regulated CD44, pSTAT3 and pNF κ B in SUM159 cells (Fig 2.8). STAT3 or NF κ B might be downstream targets of CD44 in SUM159 cell invasion. Two studies showed that CDDO-Im suppresses STAT3

phosphorylation in cancer cells. *Liby et al.* showed that CDDO-Im inhibited STAT3 phosphorylation in human myeloma and lung cancer cells, up-regulated inhibitors of STATs such as suppressor of cytokine signaling-1 and potentially down-regulated genes targeting STATs [136]. *Petronelli et al.* demonstrated that CDDO-Im inhibited STAT3 activation in ovarian cells [137]. *Yore et al.* first demonstrated CDDO-Im was a direct inhibitor of I κ B kinase β , which inhibits the binding of NF κ B to DNA and transcriptional activation [181]. Members of signal transducer and activator of transcription (STAT) family of transcription factor such as STAT3 are related to the mediation of cellular response to various cytokines and growth factors [182]. NF κ B implicates in tumor cell survival, invasion and metastasis [124, 181]. Also, STAT3 activation is associated with tumor progression in human cancers [180]. Therefore, our results are in line with the previous data that CDDO-Im may repress the activation of STAT3 and NF κ B. In the future studies, further investigation of related molecules for STAT or NF κ B pathway is needed to demonstrate CD44-STAT3 or NF κ B signaling might be a target pathway for CDDO-Im in TNBC.

2.5 Summary

Recent studies suggest that triterpenoids are potential chemopreventive agents for cancer treatment including breast cancer. In this chapter, we demonstrated that CDDO-Im inhibits cell growth in triple negative breast cancer SUM159 cells. Our findings suggest that CDDO-Im, in high dose treatment, activates the DNA damage checkpoint, G2/M arrest and induces apoptosis in SUM159 cells. In addition, the repression of CD44, pSTAT3 and pNF κ B by CDDO-Im indicated that CD44-STAT3 signaling or NF κ B pathway might be a key molecular mechanism of CDDO-Im-induced inhibition in triple negative breast cancer.

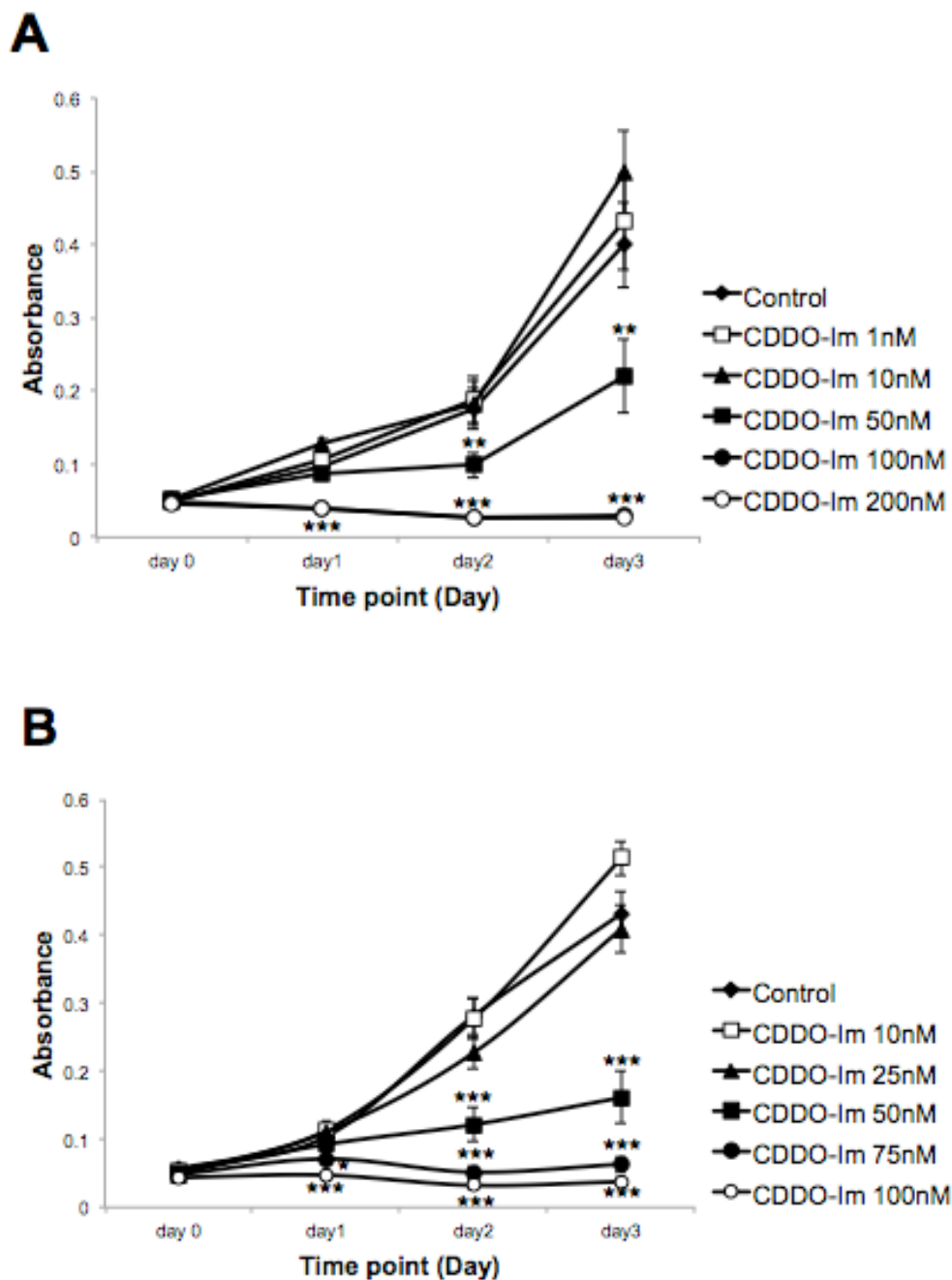


Figure 2.1 CDDO-Im inhibits cell growth in SUM159 cells. With the absence or presence of CDDO-Im at different concentration, MTT growth inhibition assay was used to measure the inhibition effect of CDDO-Im in SUM159 cells. A. SUM159 cells were treated with high range concentration of CDDO-Im B. SUM159 cells were treated with low range concentration of CDDO-Im. Error bar stands for standard error of the mean (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$, compared with control group. (n=3)

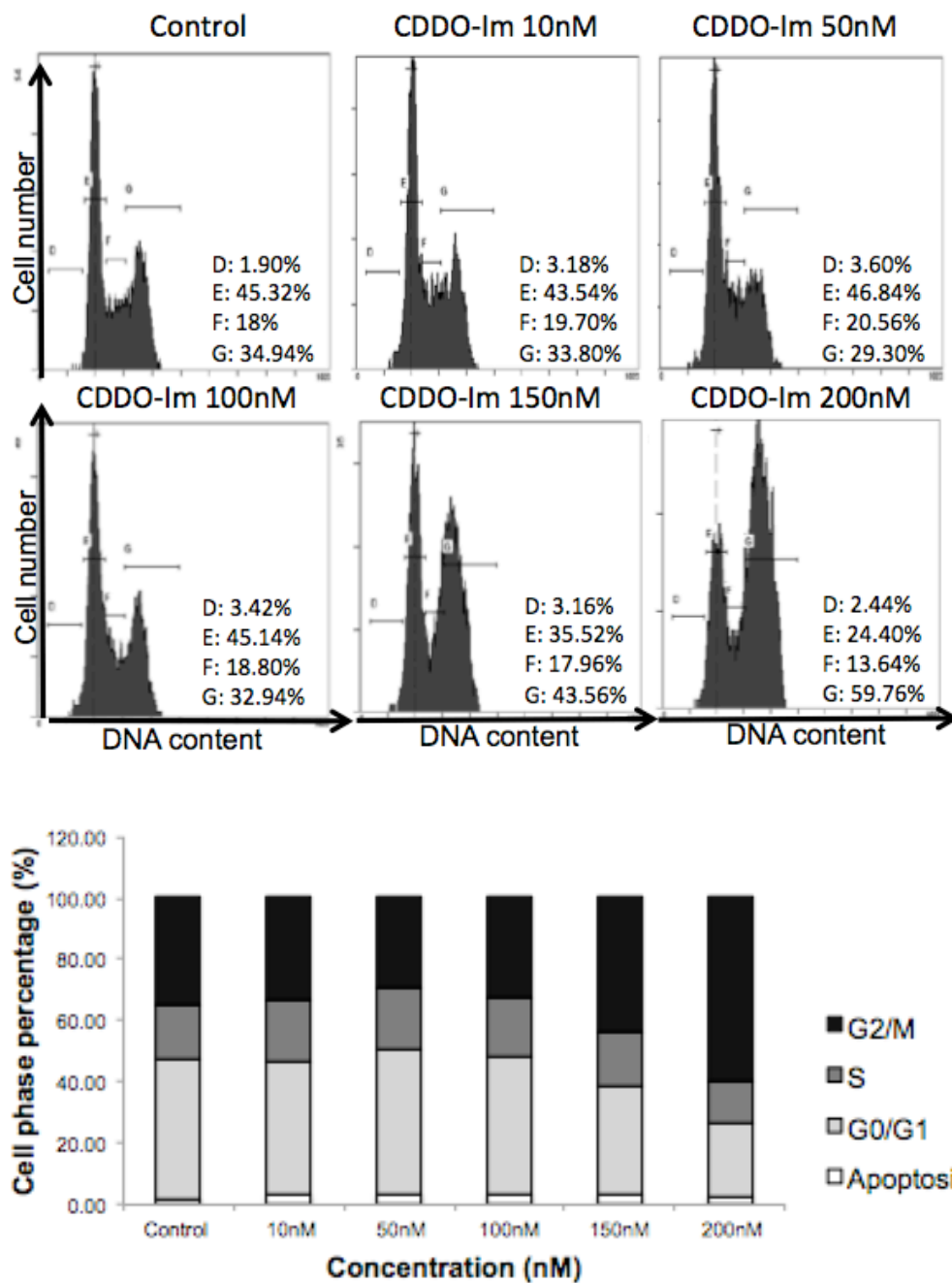


Figure 2.2 CDDO-Im induces cell cycle G2/M arrest in SUM159 cells at 24 hours. Flow cytometry analysis of SUM159 cells treated with absence or presence of 10nM, 50nM, 100nM, 150nM or 200nM CDDO-Im for 24hours. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). D phase: Apoptosis; E phase: G0/G1; F phase: S; G phase: G2/M. The experiments were repeated three times.

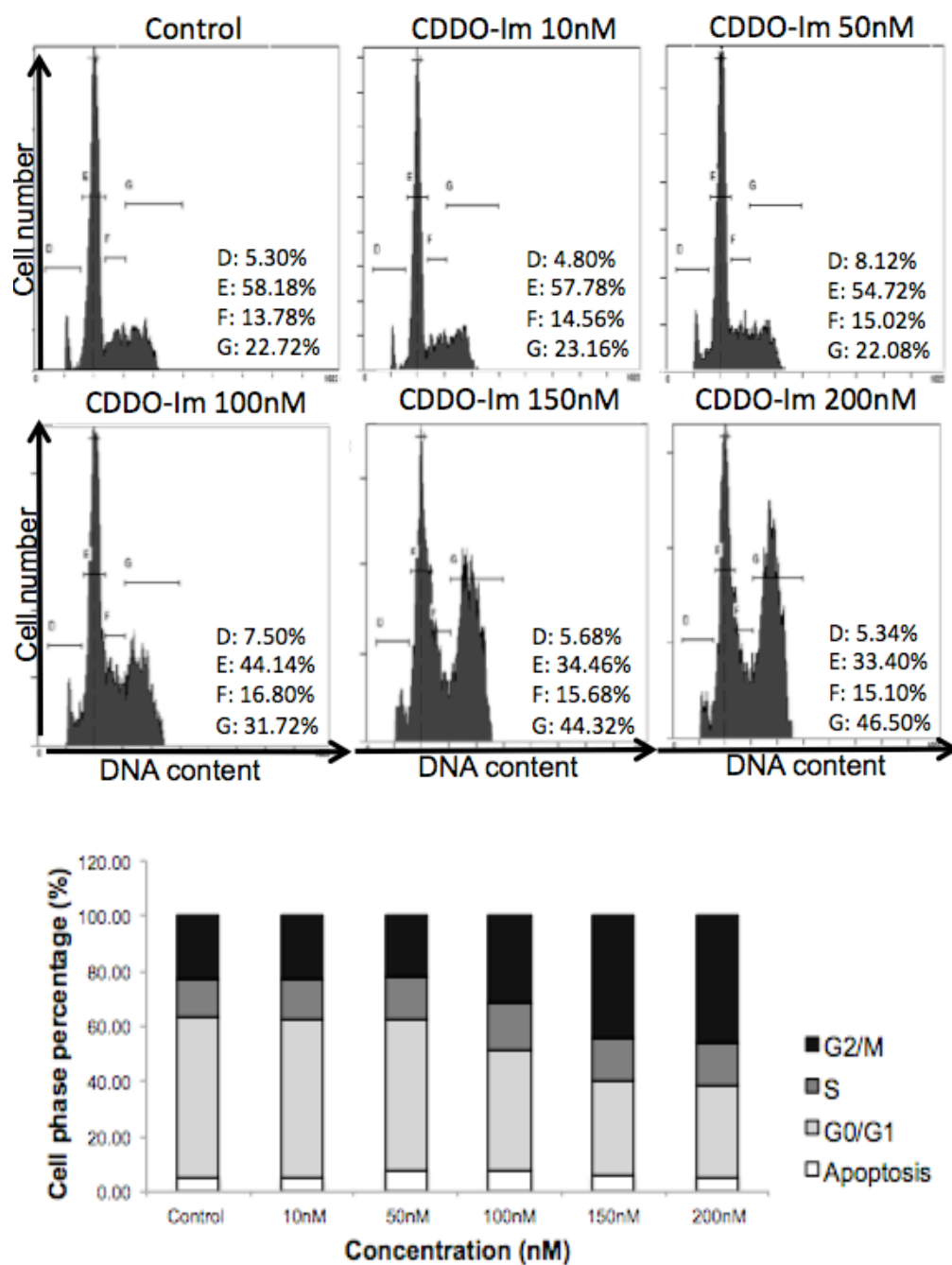


Figure 2.3 CDDO-Im induces cell cycle G2/M arrest in SUM159 cells at 48 hours. Flow cytometry analysis of SUM159 cells treated with absence or presence of 10nM, 50nM, 100nM, 150nM or 200nM CDDO-Im for 24hours. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). D phase: Apoptosis; E phase: G0/G1; F phase: S; G phase: G2/M. The experiments were repeated three times.

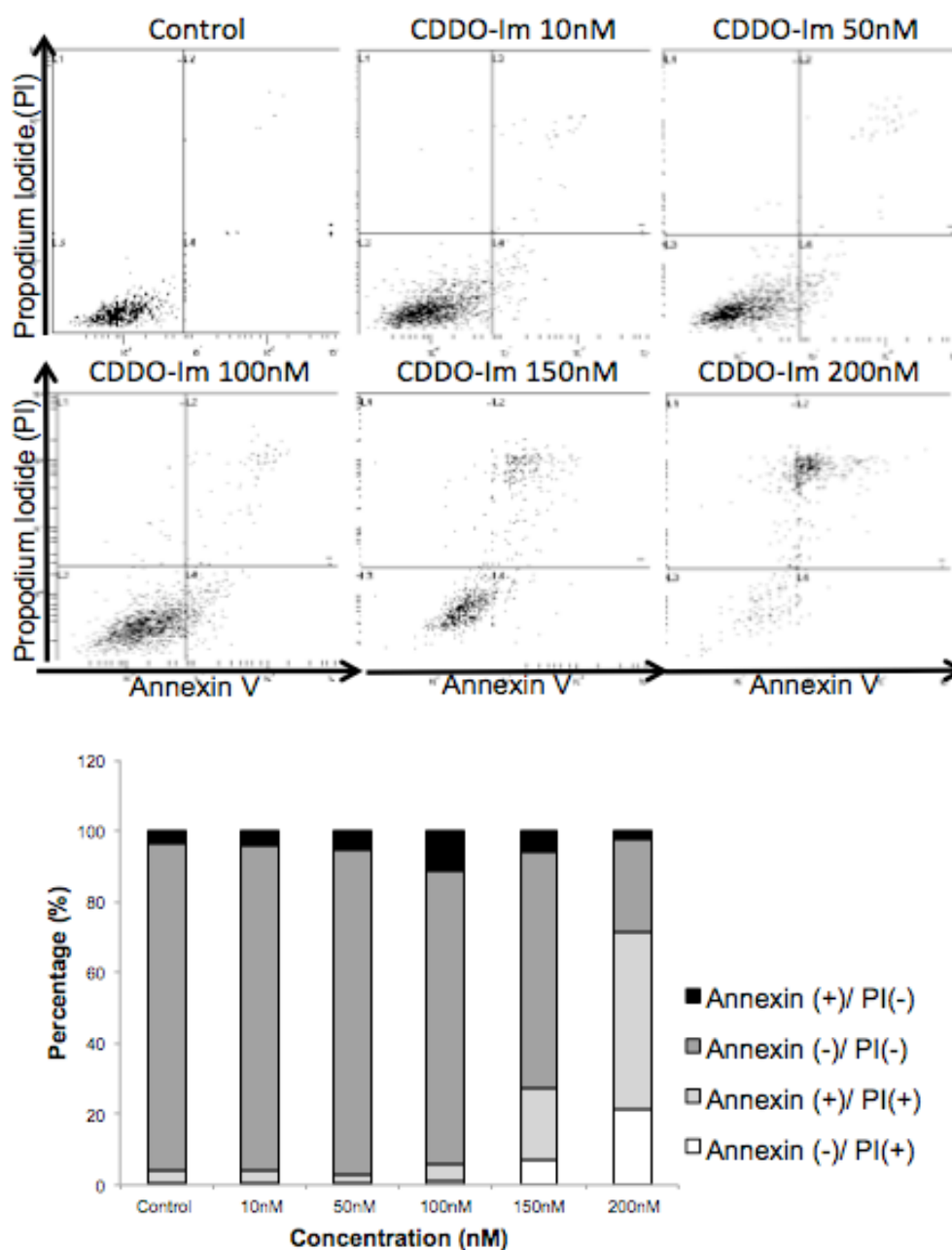


Figure 2.4 CDDO-Im induces late apoptosis in SUM159 cells. SUM159 cells were treated with CDDO-Im (10, 50, 100, 150 and 200nM) for 24 hours and were analyzed by flow cytometry for FITC-Annexin-V and propidium iodide (PI) staining. X-axis, Annexin-V; Y-axis, PI. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). The experiments were repeated three times.

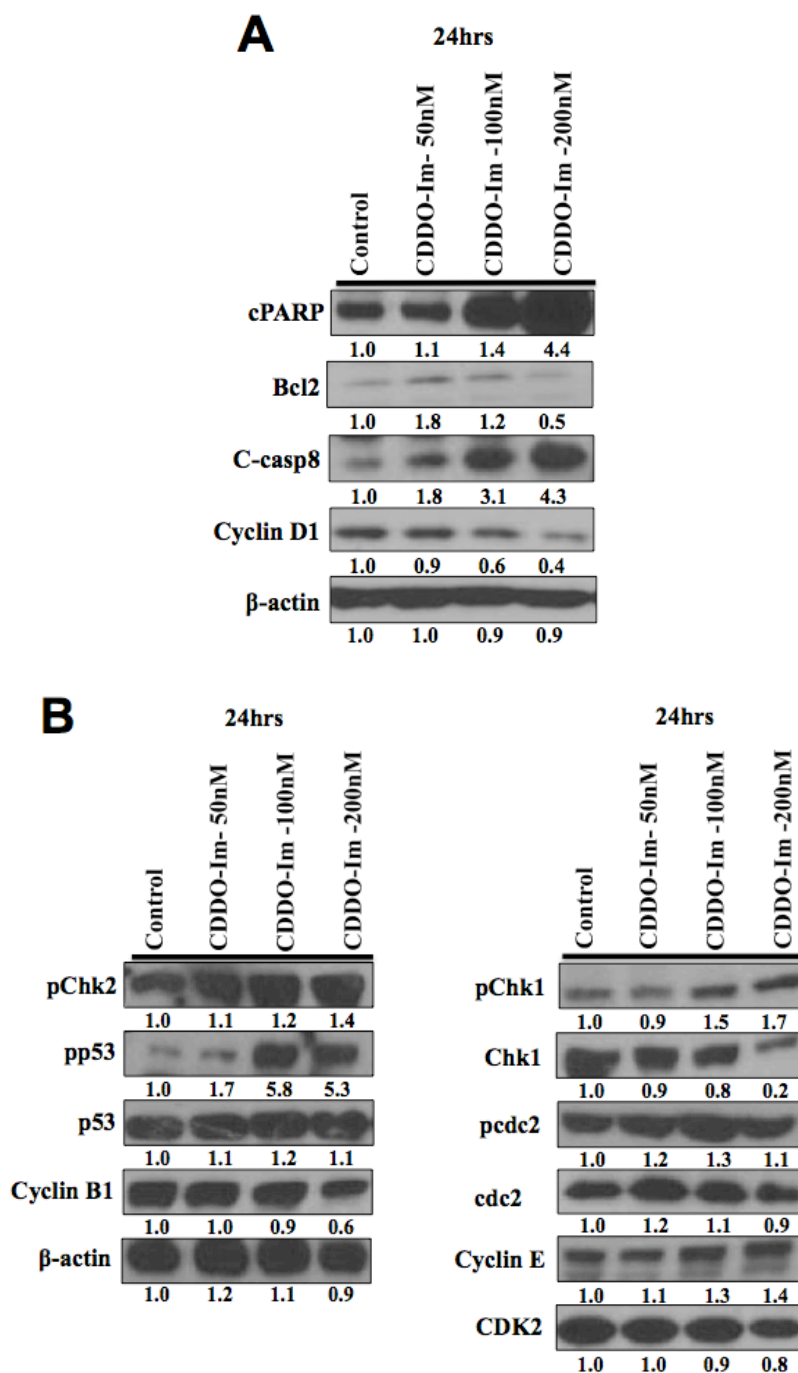


Figure 2.5 CDDO-Im inhibits cell proliferation markers and induces markers of apoptosis and G2/M arrest in SUM159 cells. SUM159 cells were treated with CDDO-Im (50, 100 or 200 nM) for 24 hours and soluble protein extracts were subjected to SDS-PAGE and Western blotting with the indicated antibodies. β -actin was used as a loading control. A. Proliferation and apoptosis markers. B. Cell cycle checkpoint markers. The experiments were repeated three times.

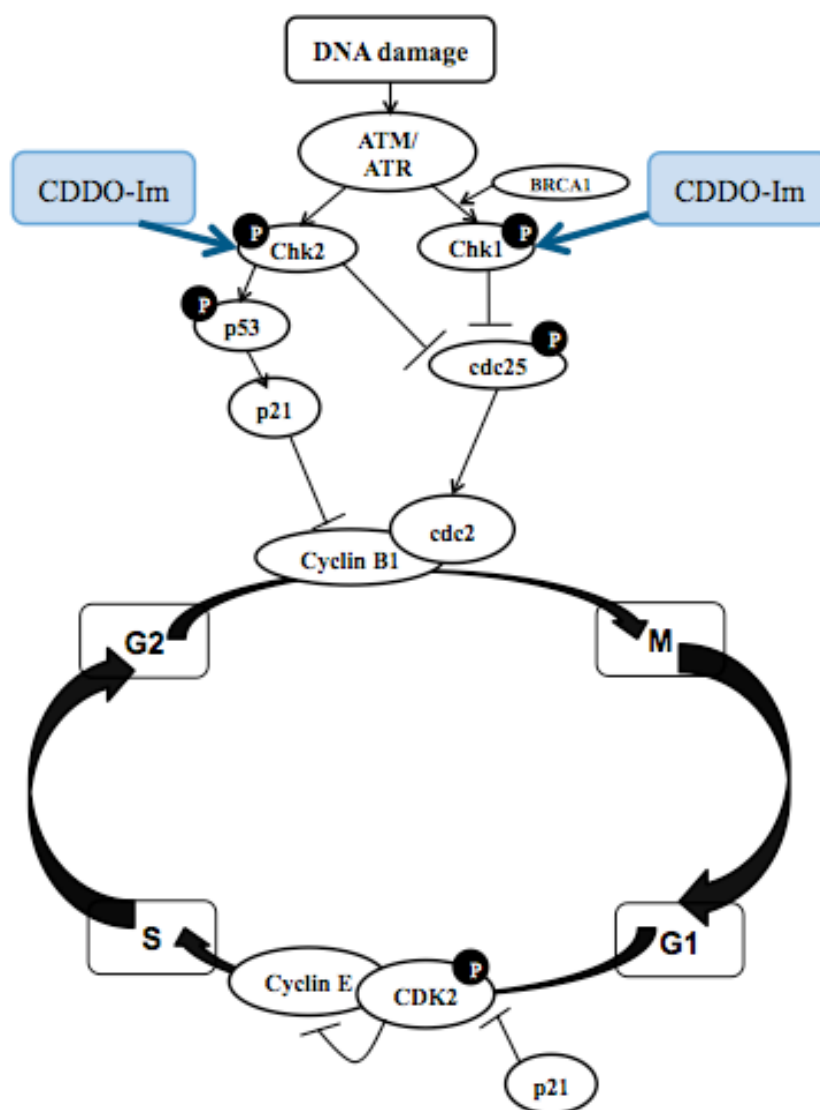


Figure 2.6 A schematic diagram of proposed mechanism of action of CDDO-Im on cell cycle G2/M arrest in triple negative breast cancer.

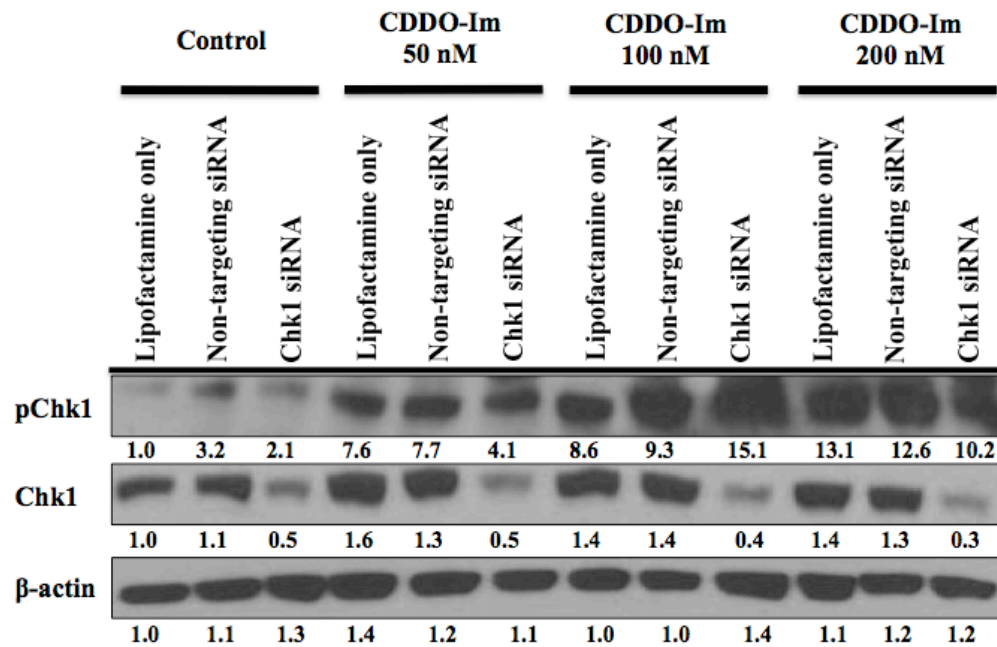


Figure 2.7 Chk1 might not be the key protein in G2/M arrest by CDDO-Im in SUM159 cells. SUM159 cells were transfected with non-target or Chk1 siRNA. After serum starvation, SUM159 cells were exposed to different concentrations (50, 100 or 200nM) of CDDO-Im for 24 hours and soluble protein extracts were subjected to SDS-PAGE and Western blotting with the indicated antibodies. β-actin was used as a loading control. The experiments were repeated two times.

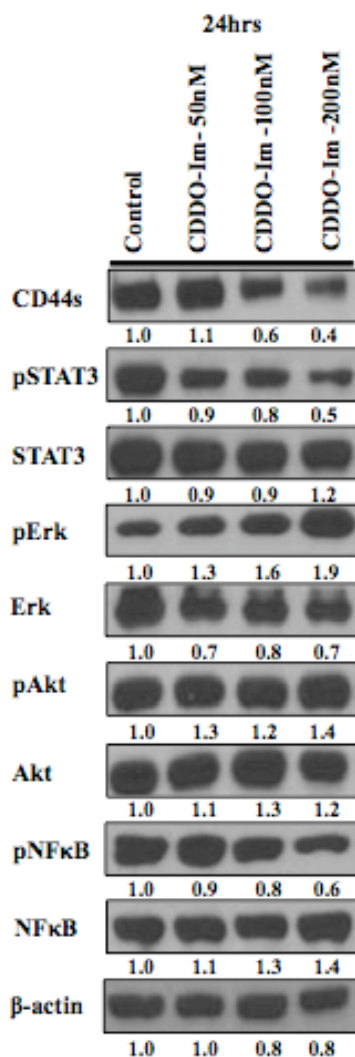


Figure 2.8 CDDO-Im decreases the protein levels of CD44, pSTAT3 and pNFκB in SUM159 cells. SUM159 cells were treated with CDDO-Im (50, 100 or 200 nM) for 24 hours and soluble protein extracts were subjected to SDS-PAGE and Western blotting with the indicated antibodies. β-actin was used as a loading control. The experiments were repeated three times.

Chapter 3: CDDO-Imidazolid (CDDO-Im) inhibits the formation of stem cell-like subpopulation of triple negative breast cancer cells

3.1 Introduction

Breast cancer generally classified into two groups based on gene expression profile of keratins: cancers that express luminal keratins or stratified epithelial keratins [158, 167, 183, 184]. Previous research classified the phenotypes of breast cancer through the cell surface expression of CD44 and CD24 [185]. *Fillmore et al.* grouped breast cancers into three phenotypes, which are based on CD44 and CD24 expression level. Luminal subtype was mainly CD24⁺ and showed more differentiated morphology and luminal cytokeratin expression [167, 185]. Basal/mesenchymal subtype was mainly CD44⁺/CD24⁻, and mixed subtype exhibited both CD44 and CD24 population [167].

In the cell model of breast cancers, there is a small subpopulation of cells, named cancer stem cells (CSCs) that contain self-sustaining cells with the ability for self-renewing and tumor maintenance [186]. CSCs define as cells that have the characteristic of self-renewal and are responsible for tumor initiation and development [187, 188]. It was first documented for leukemia and multiple myeloma [189] and it can be found in leukemia [190] and solid tumors such as blood, brain, breast and colon cancers [191-193]. Several stem cell markers including CD44, CD24, ESA, CD133 and CD90 can identify CSCs in tumors [43, 194, 195]. In human breast cancers, CSCs were likely to enrich cell subpopulations with CD44⁺/CD24^{-/low}/ESA⁺ surface markers [43, 196]. To identify CSCs, mammospheres assay is one of the strategies that used *in vitro*. Mammospheres assay can be used for quantification of stem cell/early progenitor activity and stem cell self-renewal

in normal mammary tissue and breast cancer cell lines [188]. Free-floating spheres from the assay have the characteristic of the proliferation of solid tumors and express stem-like properties when cultured under serum starvation condition [197].

In the previous chapter, we demonstrated that CDDO-Im had the inhibitory effect in SUM159 cells and decreased the protein level of CD44 *in vitro*. In this chapter, we determined whether CDDO-Im has the same inhibitory effect in breast cancer stem cells (BCSCs) subpopulation. We also evaluated the effect of CDDO-Im on cell populations in SUM159 monolayers and mammospheres. CD44, CD24 and ESA are suggestive for BCSCs markers.

3.2 Material and Methods

3.2.1 Reagents

1-[2-Cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oyl]-imidazole (CDDO-Im) was provided by Dr. Michael Sporn at Dartmouth Medical School (Hanover, NH) and dissolved in dimethyl sulfoxide (DMSO). Stock solution was prepared as 20 mM and store at -80°C refrigerator. For *in vitro* studies, stock solution was diluted with 1:1000 ratios to 20 μ M for treatment preparation.

3.2.2 Cell culture

SUM159 breast cancer cell lines were provided by Dr. Li Cai at Rutgers University. Cells were maintained in Ham's F12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, 1% HEPES solution, 1.0 μ g/ml hydrocortisone and 5.0 μ g/ml insulin at 37°C in a humidified incubator with 5% CO₂.

For mammosphere assay, SUM159 cells were maintained in complete MammoCult[®] medium, which contains MammoCult[®] Basal medium, 10% MammoCult[®] Proliferation Supplement, 0.1% fresh hydrocortisone and 0.2% Heparin (Stemcell technologies, Vancouver, Canada) at 37°C in a humidified incubator with 5% CO₂ for 7 days.

3.2.3 Mammosphere assay

SUM159 cells were washed twice with Hank's Balanced Salt Solution (HBSS), incubated with accutase for single cell suspension, centrifuged for 5 minutes at 1200 rpm, resuspend with complete MammoCult[®] medium (Stemcell technologies, Vancouver, Canada) by 25G needle syringe, and counted. Cells were plated into each well of 24-well ultra-low adherent plate (Stemcell technologies, Vancouver, Canada) (2,000 cells/well), treated with (10, 50, 100, 150 or 200 nM) or without CDDO-Im, and incubated under 5% CO₂ and 37°C condition for 7 days without moving or disturbing the plates and without replenishing the medium. After 7 days incubation, size and number of mammospheres were counted at 10X magnification, spheres greater than 60 µm are considered as mammospheres by using a microscope with graticule plate.

For developing secondary mammospheres, the entire medium culture from primary mammospheres was collected and centrifuged for 5 minutes at 1200 rpm. The pellet was mixed with 0.75 ml accutase using 25G needle and syringe to make single cell suspension. After the suspension, cells were washed with 5 ml cold HBSS, centrifuged for 5 minutes at 1200 rpm, resuspended with 1 ml complete MammoCult[®] medium and counted. Cells were plated into each well of 24-well ultra-low adherent plate and treated

with or without CDDO-Im with the same setting from the primary generation. The sample preparation was followed the same method for the primary generation.

3.2.4 Flow cytometry-double cell staining

SUM159 cells were incubated with (10, 50, 100, 150 or 200 nM) or without CDDO-Im for 24 hours. Cells were washed with PBS and then incubated with accutase for single cell suspension. After the incubation, cells were washed with stain buffer (BD bioscience, San Jose, CA), counted, centrifuged for 5 minutes at 1200 rpm, and stained with antibodies against CD44-FITC and CD24-PE (BD Pharmingen, San Jose, CA) or ESA-FITC (AbD Serotec, NC) and CD24-PE. The cells (1,000,000 cells/ 50 μ l) were incubated with antibodies for 1 hour on ice under dark condition. Unbound antibodies were washed off and cells were analyzed on a Beckman coulter gallios flow cytometer (Beckman coulter Inc., CA).

Samples for SUM159 mammospheres were collected from primary generation, which were plated into each well of multiple 6-well ultra-low adherent plates (Stemcell technologies, Vancouver, Canada) (10,000 cells/well) and incubated with (100 and 150 nM) or without CDDO-Im for 7 days. The entire culture were collected and centrifuged for 5 minutes at 1200 rpm. The pellet was added with 0.75 ml accutase and used 25G needle and syringe to make single cell suspension. After the suspension, cells were washed with stain buffer (BD bioscience, San Jose, CA) and followed the same procedure as for preparing SUM159 monolayers. SUM159 mammospheres were stained with antibodies against CD44-FITC and CD24-PE (BD Pharmingen, San Jose, CA) or

ESA-FITC (AbD Serotec, NC) and CD24-PE and were analyzed on a Beckman coulter gallios flow cytometer (Beckman coulter Inc., CA).

3.2.5 *In vitro* experiment design

SUM159 cells were taken from the nitrogen tank and passed. After at least 10 passages in Ham's F12 medium, a stable cell set, which checked with cell cycle analysis, was obtained and then used for *in vitro* studies. Based on the results from previous experiments, double cell staining was followed the same treatment setting as cell cycle analysis and apoptosis assay for 24 hours. Cells were stained with human cell surface markers: ESA, CD44 and CD24 as described above.

For mammosphere assay, cells were treated with the same treatment setting as previous experiments to examine the growth inhibitory effect of CDDO-Im in stem cell-like breast cancer cells. Two concentrations of CDDO-Im were selected and treated for 7 days. After 7 days incubation, cells were collected for double cell staining analysis.

3.2.6 Statistical analysis

Statistical significance was evaluated using the Student's *t* test or one-way analysis of variance (ANOVA).

3.3 Results

3.3.1 SUM159 monolayers show basal-like phenotype

Fillmore et al. demonstrated that basal/mesenchymal subtype cells such as MDA-MB-231, SUM159 and SUM1315 cells were mainly CD44⁺/CD24⁻ population [167]. To

further examine the cell population in SUM159 cells, we examined the expression level of ESA, which was used to identify epithelial cancer cells from benign reactive mesothelial cells [43]. SUM159 cells were mainly CD44⁺/CD24⁻, but nearly 20% were exhibited CD44⁺/CD24⁺ population (Fig 3.1A). Cells were mainly ESA⁻/CD24⁻, but nearly 10% of the cells were ESA⁻/CD24⁺ population (Fig 3.1B). Therefore, the phenotype of SUM159 cells was mainly CD44⁺/CD24⁻/ESA⁻, which is consistent with basal cell type identification.

3.3.2 CDDO-Im reduces CD44⁺/CD24⁻ population without changing ESA⁻ population in SUM159 monolayers

We tested the effect of CDDO-Im on the cell population in SUM159 monolayers. SUM159 monolayers were treated with the same setting as cell cycle analysis and apoptosis assay and were co-stained with CD44 and CD24 or CD24 and ESA by flow cytometry. There were no percentage changes in entire CD44/CD24 cell population within CDDO-Im treatment (Fig 3.2). However, in high dose treatment such as 200 nM, cells changed its population from CD44⁺/CD24⁻ to CD44^{+/hi}/CD24⁺, which means SUM159 monolayers had less CD44⁺ population and more CD24⁺ population (Fig 3.2).

In Fig 3.3, there was no significant change in CD24/ESA population by the treatment with CDDO-Im in SUM159 monolayers. SUM159 monolayers mainly showed CD24⁻/ESA⁻ population in each groups; however, SUM159 monolayers had slightly increased CD24⁻ and ESA⁺ population in high dose treatment (200 nM) (Fig 3.3).

3.3.3 SUM159 mammospheres present breast cancer stem cell phenotype

BCSCs have the characteristic of self-renewal [187], and they are related to tumor initiation and development [188]. Mammosphere assay was used to quantify stem cell/early progenitor activity and stem cell self-renewal in normal mammary tissue and breast cancer cell lines [188]. SUM159 mammospheres were co-stained with CD44/CD24 or CD24/ESA by flow cytometry. SUM159 mammospheres had regular circle shape and mostly were greater than 200 μm (Fig 3.4A). Compared to SUM159 monolayers, SUM159 mammospheres were mainly CD44^{+/hi}/CD24⁻/ESA⁺ (Fig 3.4B and C), which present CSCs profile [196]. We also compared the profile of primary and secondary mammospheres. Primary and secondary mammospheres showed mainly CD44^{+/hi}/CD24⁻/ESA⁺, but secondary mammospheres had more concentrated expression level of CD44⁺ and less expression level of ESA⁺ and CD24⁺ (Fig 3.5A and B).

3.3.4 CDDO-Im reduces the number and size of SUM159 mammospheres in a dose dependent manner

To determine whether CDDO-Im has the growth inhibitory effect on cancer stem cell subpopulation, we treated SUM159 mammospheres with CDDO-Im (10, 50, 100, 150 or 200 nM) for 7 days and evaluated the number and size of primary and secondary mammospheres.

Primary mammospheres were mainly greater than 200 μm in the control group, but there was no difference between control and low dose treatment (Fig 3.6). The size of primary mammospheres was decreased in a dose dependent manner (Fig 3.6). Secondary mammospheres had more number and greater size than primary ones in the control group

(Fig 3.7). There were no significant size change in low dose treatment such as 10 and 50 nM, and secondary mammospheres were still greater than 200 μ m in 100 nM group (Fig 3.7). The size of secondary mammospheres was significant reduced in high dose treatments (Fig 3.7).

We also found that the number of primary mammospheres was decreased in a dose dependent manner (Fig 3.8A), but number of secondary mammospheres was increased in low dose treatment such as 10 and 50 nM and decreased in high dose treatment such as 100, 150 and 200 nM (Fig 3.8B). We calculated the mammosphere forming efficiency (MFE) (%), which followed this formula: (number of mammospheres per well/number of cells seeded per well) X100 [188]. The MFE of primary mammospheres in control group was 1.7%, whereas the MFE of secondary mammospheres in control group was 2.32% (Fig 3.8). CDDO-Im reduced MFE in a dose dependent manner, and MFE was less than 1% in high dose treatments (Fig 3.8). We also calculated mammosphere self-renewal for control groups, which used this formula: total number of secondary mammospheres formed/ total number of primary mammospheres formed [188]. The mammosphere self-renewal in control groups was 1.35, which means cells in every mammosphere can self-renewal and may form more mammospheres. Collectively, CDDO-Im reduced the number and the size of primary and secondary mammospheres in a dose dependent manner. Secondary mammospheres may be more resistant to the treatment with CDDO-Im than primary mammospheres.

3.3.5 CDDO-Im reduces CD44⁺/CD24⁻/ESA⁺ population in SUM159 mammospheres

Next, we tested the effect of CDDO-Im on the cell population in mammospheres. Mammospheres were treated with 100 nM and 150 nM for 7 days based on previous results and were co-stained with CD44/ CD24 or CD24/ ESA for flow cytometry. Cell population of mammospheres was significantly changed from CD44⁺/CD24⁻ to CD44⁻/^{hi}/CD24^{+/hi}, which means mammospheres had less expression of CD44 and more expression of CD24 population (Fig 3.9).

Mammospheres mainly showed ESA⁺/CD24⁻ population in the control group. CDDO-Im reduced ESA⁺/CD24⁻ population and increased ESA⁻/CD24⁻ population in 100 nM group (Fig 3.10). In high dose treatment with CDDO-Im, mammospheres mainly expressed ESA⁺/CD24⁺ population (Fig 3.10). Collectively, CDDO-Im changed the cell population in mammospheres from CD44^{+/hi}/CD24⁻/ESA⁺ to CD44⁻/^{hi}/CD24^{+/hi}/ESA⁻.

3.4 Discussion

Cancers develop from cells with self-renewal characteristics are analogous to stem cells [186]. *Fillmore et al.* suggested breast cancer cell lines with self-renewing tumorigenic cells are identical models to study CSCs [167]. CSCs can be identified by several cell surface markers including CD44, CD24, ESA [43], CD133 [194] and CD90 [195], either singly or in combination. CD44⁺ phenotype correlates with colon, breast, prostate, and pancreatic cancer initiator cells [43, 192, 198, 199]. CD24⁺ phenotype is shown in pancreatic cancer [199] but in breast cancer is shown as CD24⁻ phenotype [43]. ESA⁺ phenotype expresses in pancreatic and breast cancer [43, 199]. CD133⁺ phenotype was discovered in colon, prostate, pancreatic, and brain cancer [192, 198-200]. CD90⁺

phenotype expressed in bone marrow-derived mesenchymal stem cells and hepatic stem/progenitor cells [195].

In our study, we examined CSC characteristic in SUM159 monolayers and mammospheres using CD44, CD24 and ESA as stem cell markers. Monolayers mostly exhibited CD44⁺/CD24⁻/ESA⁻ phenotype (Fig 3.1), while mammospheres exhibited CD44⁺/CD24⁻/ESA⁺ phenotype (Fig 3.4), which is consistent with previous studies [43, 167]. In human breast cancers, CD44⁺/CD24⁻ cells have been reported to have stem/progenitor properties and involve in invasiveness and tumorigenicity [43]. CD44⁺/CD24⁻ cells are mostly related to cell motility and angiogenesis [201]. These cells exhibit basal-like subtypes [167]. Therefore, SUM159 cells might have the characteristic of CSCs.

To investigate whether CDDO-Im have the effect on the cell population, we examined the cell profile on treated cells. We found that the cell population of monolayers shifted to CD44^{+/hi}/CD24^{-/hi} phenotype and mammospheres shifted to CD44^{-/hi}/CD24^{+/hi}/ESA⁻ phenotype under high dose treatment with CDDO-Im (Fig 3.2, 3.3, 3.9 and 3.10). CDDO-Im also reduced the number and size of mammospheres in a dose dependent manner (Fig 3.6-3.8). These results clearly showed that CDDO-Im inhibited the growth of mammospheres and reduced the percentage of stem cell subpopulation.

Triterpenoids are phytochemicals and several of them were reported to have anti-tumorigenic activities *in vivo* [90, 202]. However, studies with the anti-CSCs effect of natural and synthetic triterpenoids are limited. Methyl Antecinate A (MAA), an ergostane triterpenoid from *Antrodia camphorate*, could inhibit cell migration ability and mammospheres forming capability in MCF7 breast cancer cells [203]. Lup-20(29)-en-3β-

ol (lupeol), a triterpenoid found in fruits and vegetables, could target tumor-initiating cells of liver cancer [139]. Lupeol repressed hepatic spheres formation in hepatocellular carcinoma (HCC) cells, inhibited tumorigenicity in nude mice and down-regulated CD133 expression [139]. Several studies also showed anti-CSCs effect in breast cancer by other natural compounds such as curcumin, piperine [204] and sulforaphane [205, 206]. Curcumin and piperine inhibited mammosphere formation and reduced the expression level of aldehyde dehydrogenase, a breast stem cell marker, in breast cancer [204]. Sulforaphane, a compound from broccoli, inhibited mammospheres forming capability and down-regulated the Wnt/ β -catenin self-renewal pathway [205].

Previous studies investigated the anti-CSC effect mostly focus on natural triterpenoids or other natural compounds, and studies with synthetic triterpenoids are limited. Our study demonstrated that CDDO-Im inhibited mammosphere formation and reduced the expression of $CD44^+/CD24^-/ESA^+$, suggesting that CDDO-Im might have a potential as chemopreventive agents targeting cancer stem cells. Future experiments will involve deciphering the mechanism used by synthetic triterpenoids for anti-CSC effect in TNBC.

3.5 Summary

SUM159 monolayers highly express $CD44^+/CD24^-/ESA^-$ population and SUM159 mammospheres highly express $CD44^+/CD24^-/ESA^+$ population *in vitro*. In this chapter, we demonstrated that CDDO-Im reduced the formation of CSC subpopulation in SUM159 monolayers and mammospheres. Our study suggests CDDO-Im as a potentially useful agent for inhibiting CD44 and ESA expressing CSCs in breast cancer.

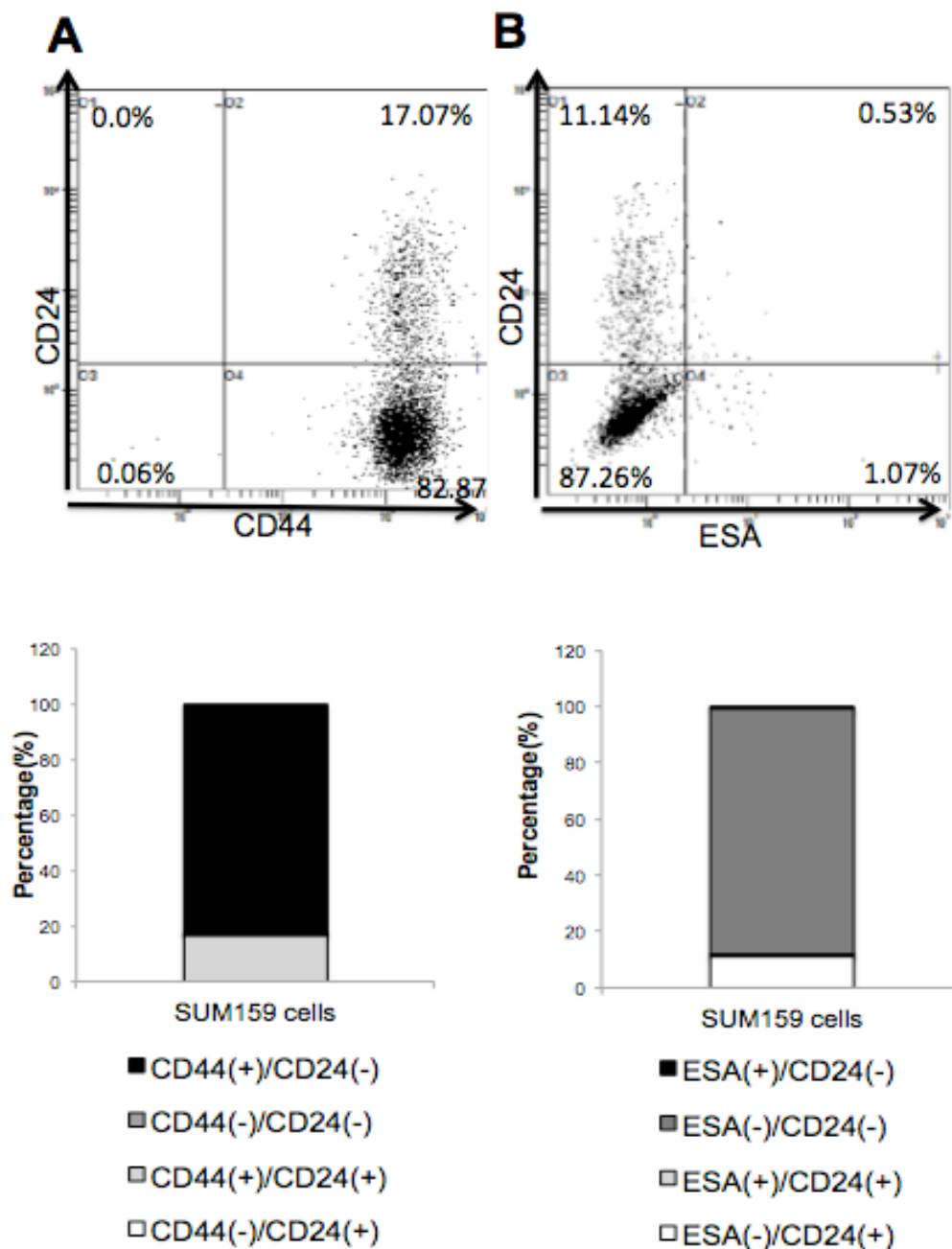


Figure 3.1 SUM159 monolayers show basal-like phenotype. To identify the expression profile of SUM159 cells, cells were analyzed by flow cytometry for A. FITC-CD44 and PE-CD24 staining and B. FITC-ESA and PE-CD24 staining. X-axis, A. CD44 B. ESA; Y-axis, CD24. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). The experiments were repeated three times.

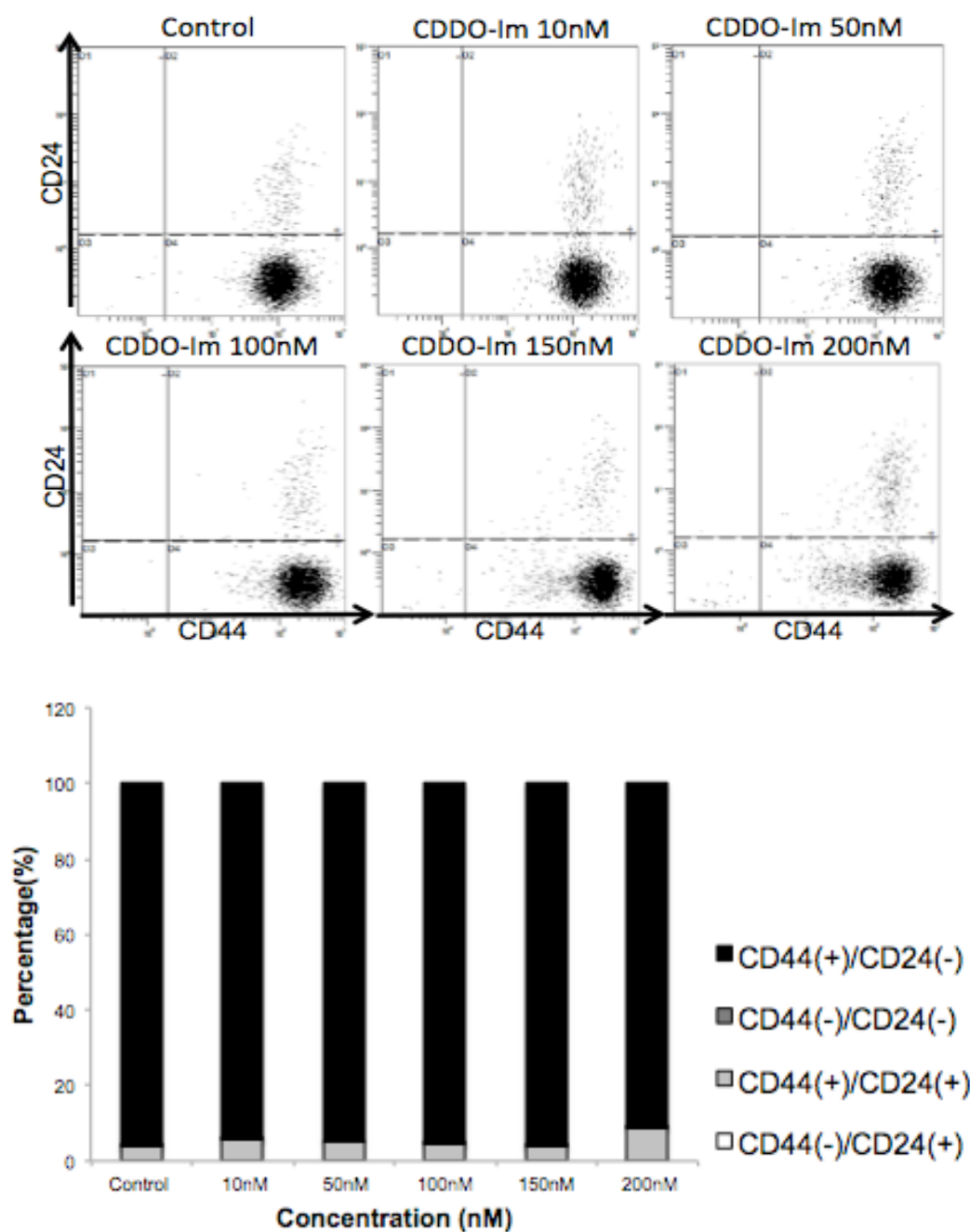


Figure 3.2 CDDO-Im reduces the expression level of CD44⁺ and induces the expression of CD24⁺ in SUM159 monolayers. SUM159 cells were treated with CDDO-Im (10, 50, 100, 150 and 200nM) for 24 hours and were analyzed by flow cytometry for FITC-CD44 and PE-CD24 staining. X-axis, CD44; Y-axis, CD24. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). The experiments were repeated three times.

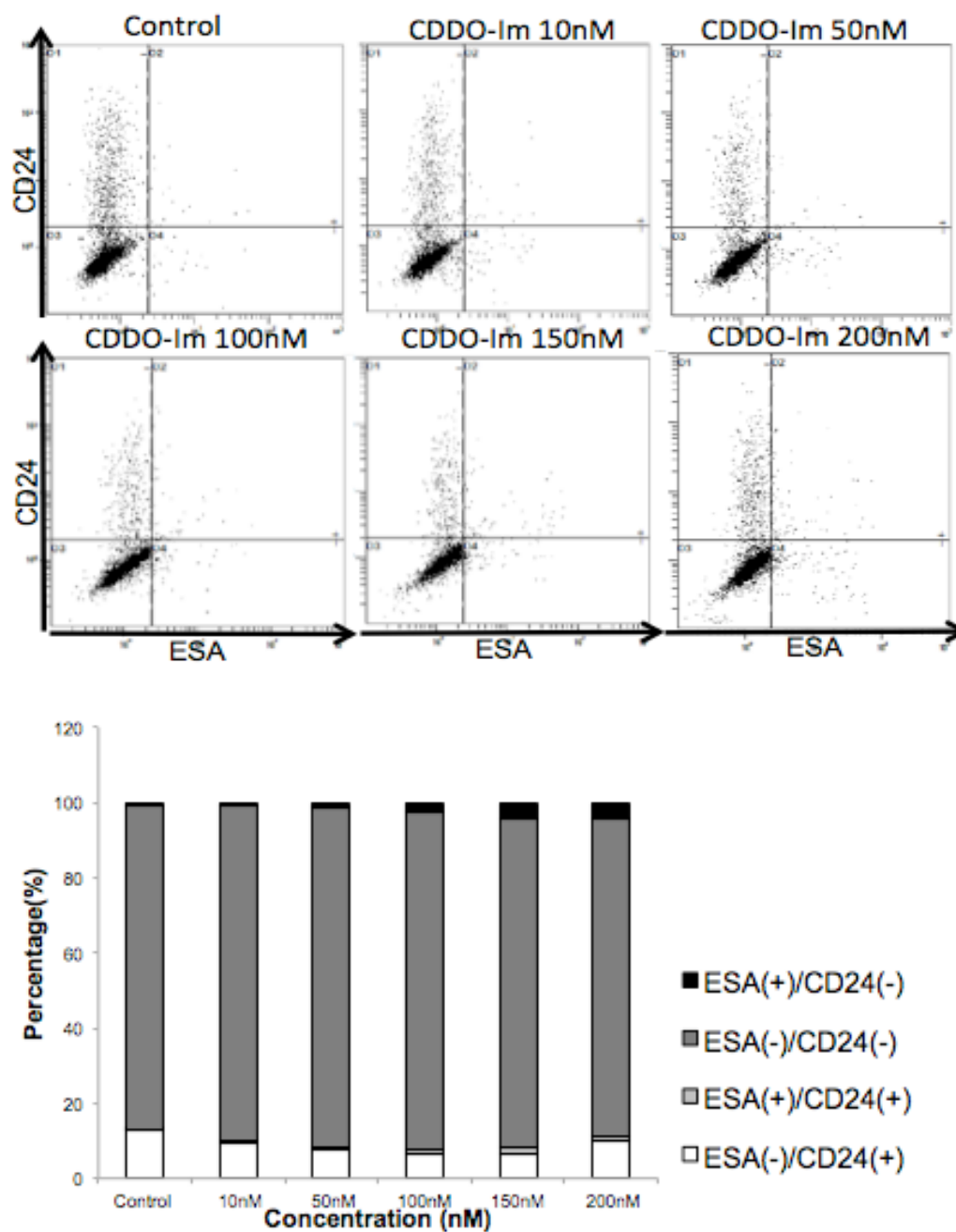


Figure 3.3 CDDO-Im does not change the expression level of ESA in SUM159 monolayers. SUM159 cells were treated with CDDO-Im (10, 50, 100, 150 and 200nM) for 24 hours and were analyzed by flow cytometry for FITC-ESA and PE-CD24 staining. X-axis, ESA; Y-axis, CD24. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). The experiments were repeated three times.

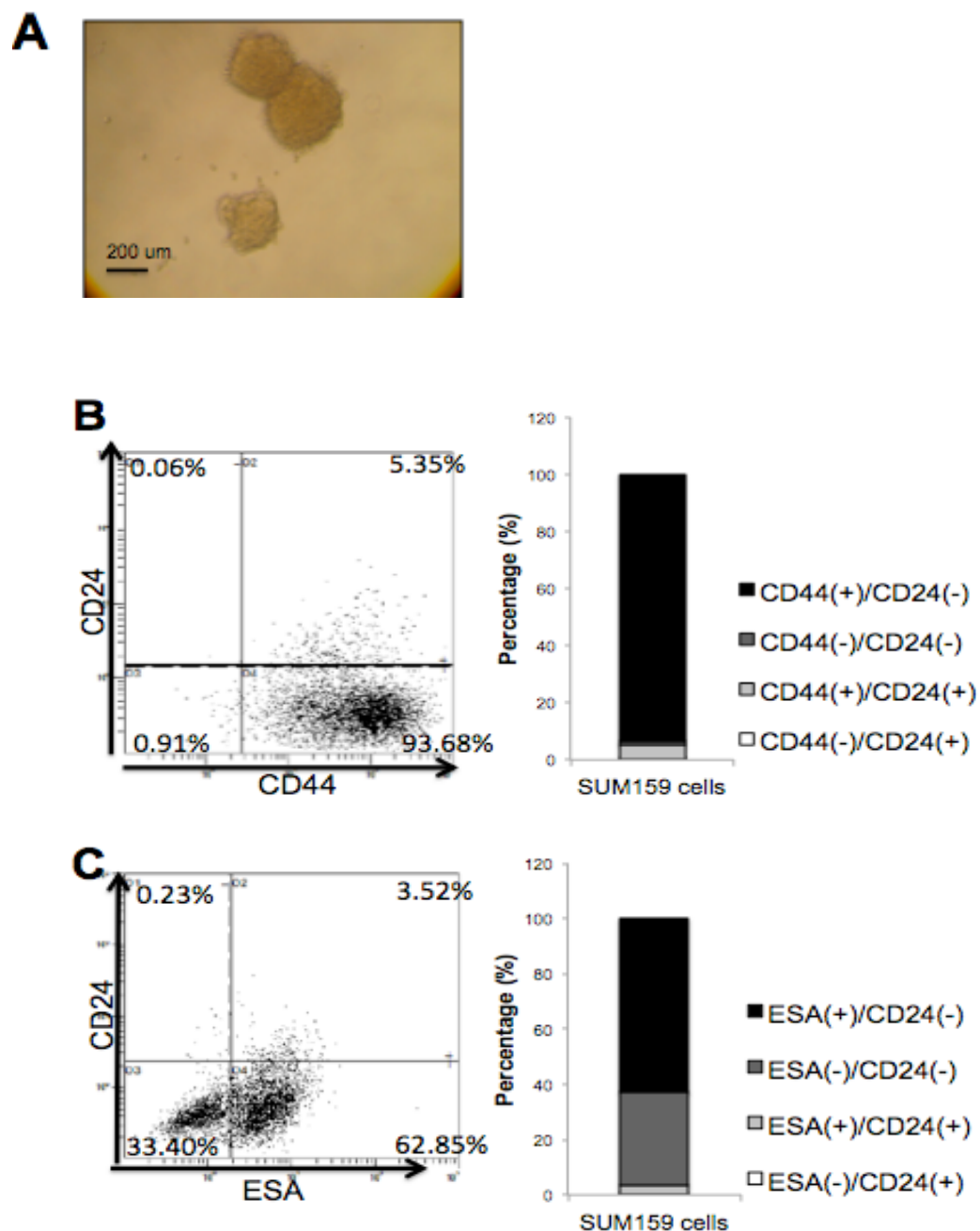


Figure 3.4 The profile of SUM159 mammospheres. A. Phase-contrast image of SUM159 mammospheres at day 7 under a 10X objective. B and C. Mammospheres were analyzed by flow cytometry for B. FITC-CD44 and PE-CD24 staining and C. FITC-ESA and PE-CD24 staining. X-axis, B. CD44 C. ESA; Y-axis, CD24. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). The experiments were repeated three times.

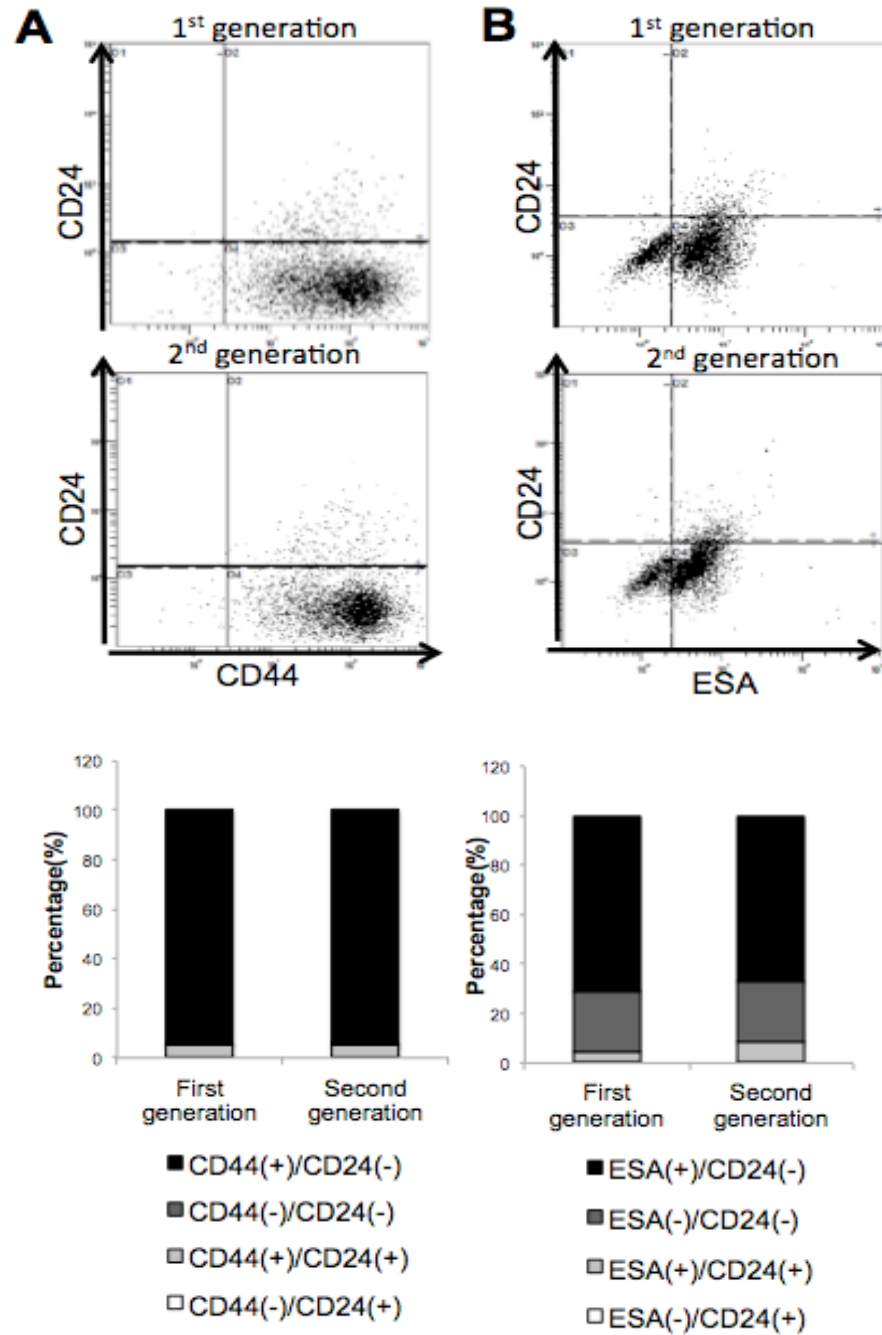


Figure 3.5 Primary and secondary SUM159 mammospheres show breast cancer stem cell phenotype. SUM159 mammospheres were analyzed by flow cytometry for A. FITC-CD44 and PE-CD24 staining and B. FITC-ESA and PE-CD24 staining. X-axis, A. CD44 B. ESA; Y-axis, CD24. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). The experiments were repeated three times.

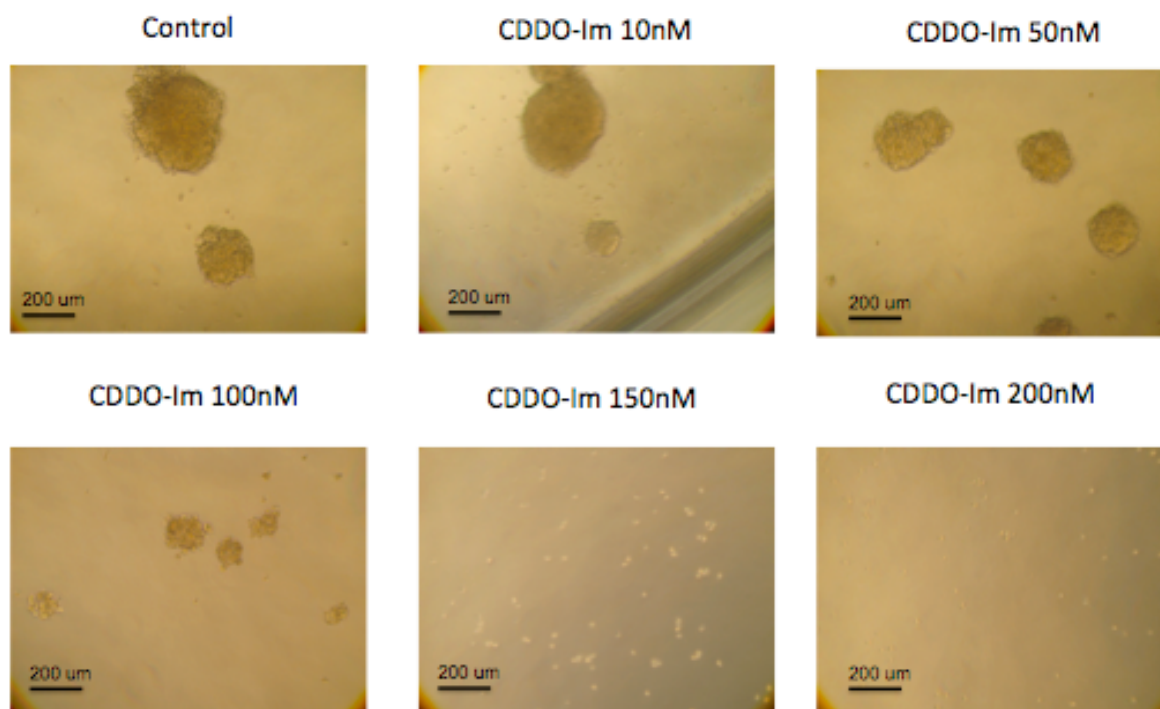


Figure 3.6 CDDO-Im reduces the size of primary SUM159 mammospheres in a dose dependent manner. For SUM159 cells, 2,000 single cells/well were plated on ultra-low adherence 24 well plates and spheres visible under microscope were counted at day 7. Spheres larger than 60μm were considered as mammospheres. Photo shows the image of mammospheres at day 7 under a 10X objective. The experiments were repeated three times.

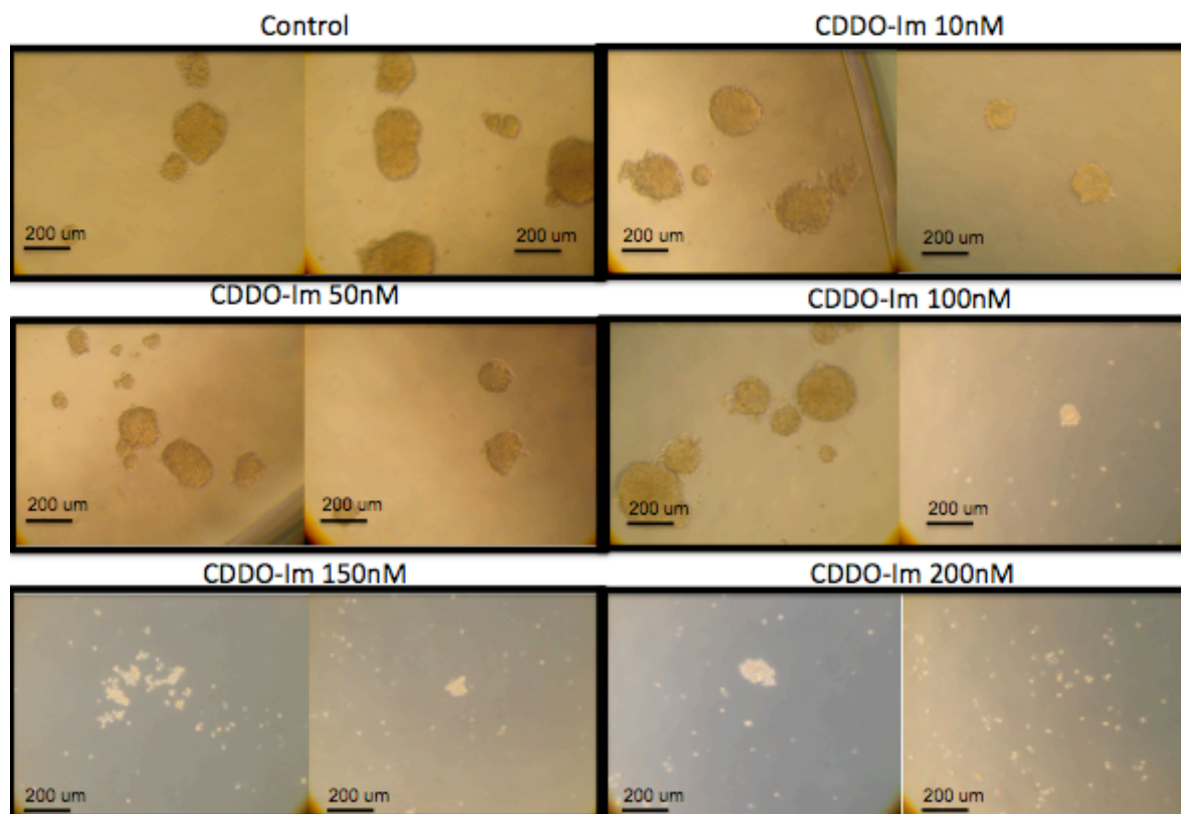


Figure 3.7 CDDO-Im reduces the size of secondary SUM159 mammospheres in a dose dependent manner. For SUM159 cells, 2,000 single cells/well were plated on ultra-low adherence 24 well plates and spheres visible under microscope were counted at day 7. Spheres larger than 60 μ m were considered as mammospheres. Photo shows the image of mammospheres at day 7 under a 10X objective. The experiments were repeated three times.

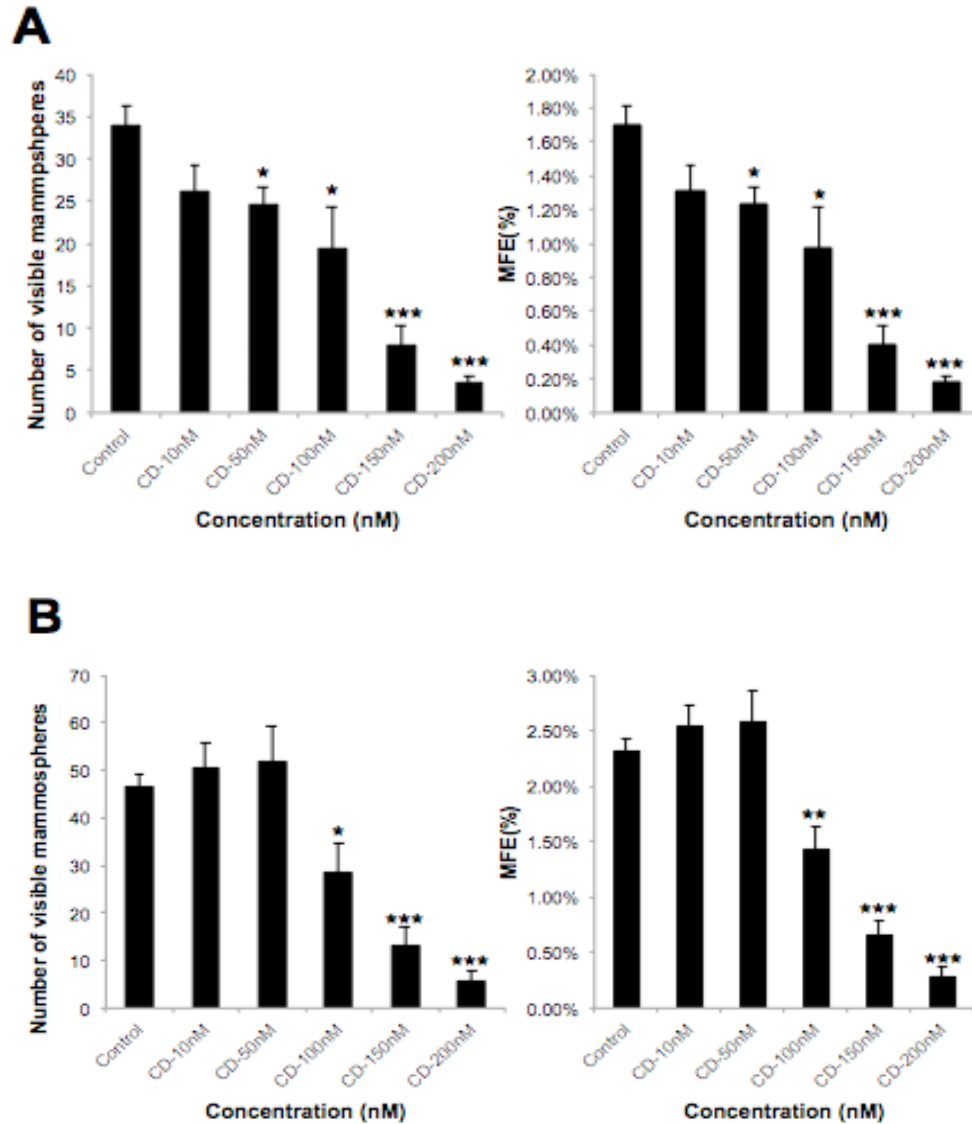


Figure 3.8 CDDO-Im reduces the number of SUM159 mammospheres in a dose dependent manner. For SUM159 cells, 2,000 single cells/well were plated on ultra-low adherence 24 well plates and spheres visible under microscope were counted at day 7. Spheres larger than 60 μ m were considered as mammospheres. Mammosphere forming efficiency (MFE) and mammosphere self-renewal rate were calculated. A. Numbers and MFE of primary mammospheres. B. Numbers and MFE of secondary mammospheres. Error bar stands for standard error of the mean (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$, compared with control group. (n=3)

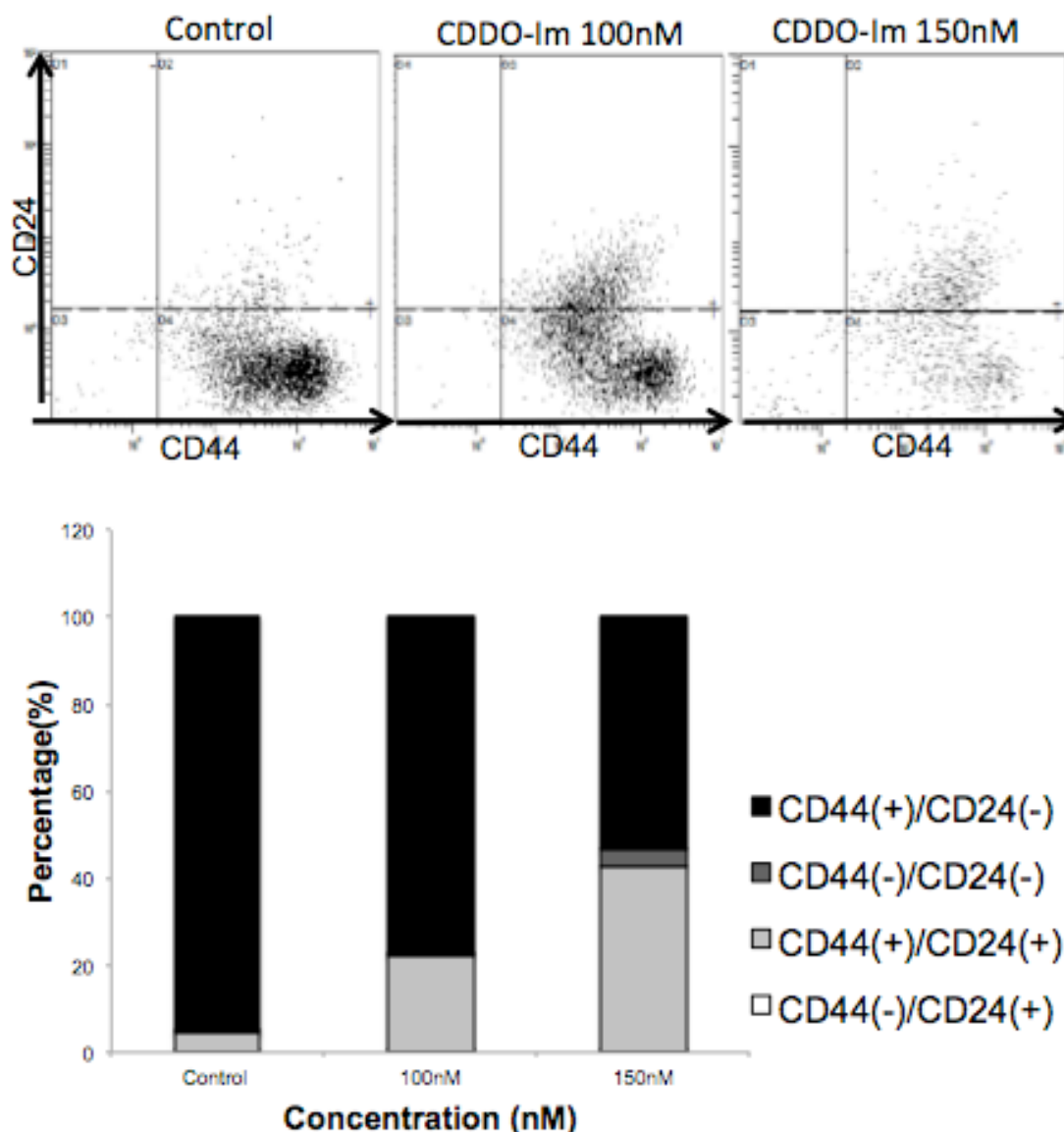


Figure 3.9 CDDO-Im reduces the expression level of CD44⁺/CD24⁻ in SUM159 mammospheres. SUM159 mammospheres were treated with CDDO-Im (100 and 150nM) for 7 days and were analyzed by flow cytometry for A. FITC-CD44 and PE-CD24 staining and. X-axis, CD44; Y- axis, CD24. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). The experiments were repeated three times.

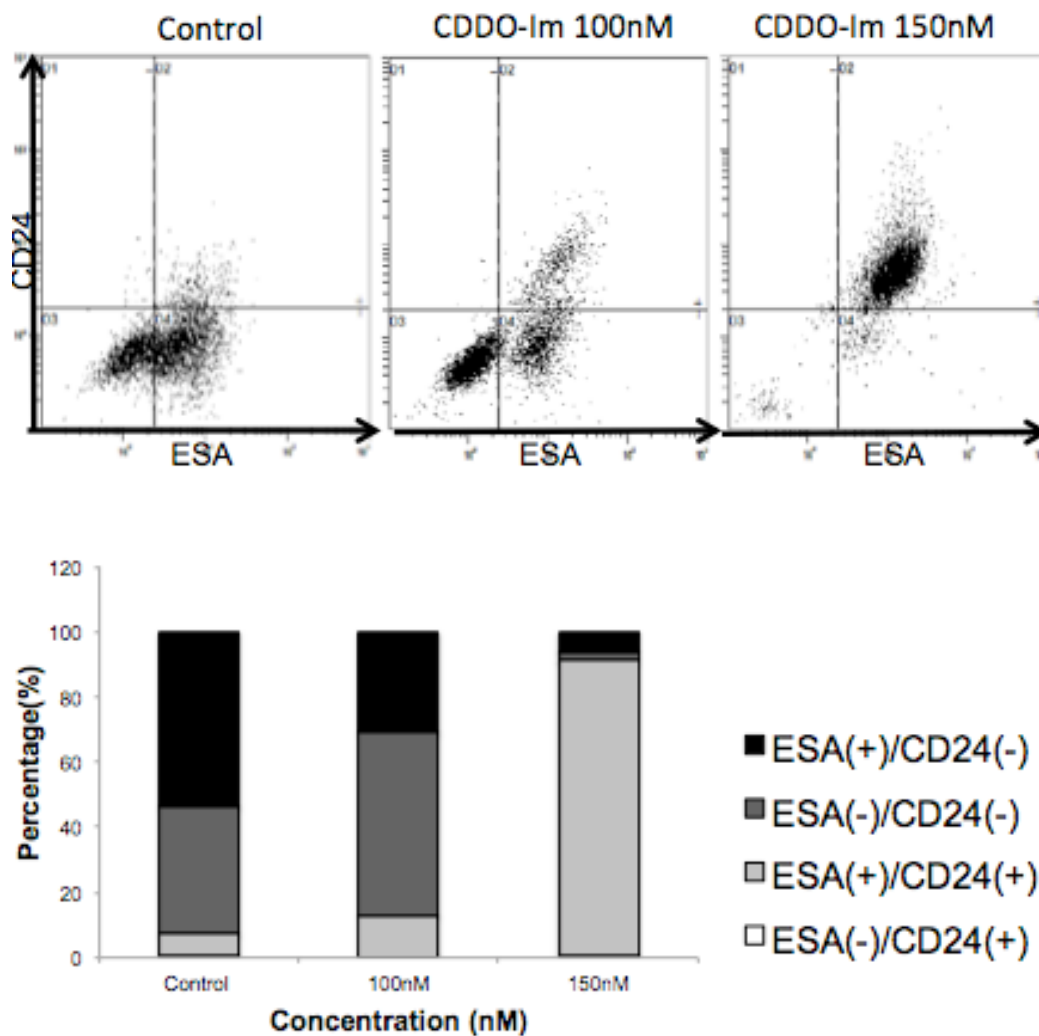


Figure 3.10 CDDO-Im reduces the expression level of ESA⁺/CD24⁻ in SUM159 mammospheres. SUM159 mammospheres were treated with CDDO-Im (100 and 150nM) for 7 days and were analyzed by flow cytometry for FITC-ESA and PE-CD24 staining. X-axis, ESA; Y-axis, CD24. Samples were analyzed by using Beckman coulter gallios flow cytometer (EOSHI. Rutgers). The experiments were repeated three times.

Conclusion

Triple negative breast cancer is not a common breast cancer among women but is the most aggressive type of breast cancer. Recent studies investigated several chemopreventive agents including triterpenoids as potent anti-cancer treatment *in vitro* and *in vivo*. We have investigated the effect of CDDO-Im, a synthetic triterpenoid, in SUM159 monolayers as well as mammospheres. CDDO-Im inhibits the growth of SUM159 cells and activates DNA damage checkpoint that leads to G2/M arrest and apoptosis in high dose treatment. CDDO-Im down-regulated the protein level of CD44, pSTAT3 and pNF κ B in SUM159 cells. CDDO-Im reduces the number and size of mammospheres in a dose dependent manner. In a further investigation of cell population in SUM159 monolayers and mammospheres, we found that CDDO-Im both reduced the expression level of CD44, a key marker of breast CSCs, in SUM159 monolayers and mammospheres in a dose dependent manner. The cell population had less BCSC profile, which refers to CD44⁺/CD24⁻/ESA⁺, in both monolayers and mammospheres from SUM159 cells. Overall, these results suggest CDDO-Im may be considered as a potent agent for treatment of triple negative breast cancer.

Future direction

***In vitro* studies**

In the first part, we demonstrated the inhibitory effect of CDDO-Im in SUM159 cells and further examined with related protein markers on cell proliferation, apoptosis and cell cycle checkpoint. In siRNA analysis, we found that Chk1 may not be the key protein of G2/M arrest mechanism. To confirm CDDO-Im induces G2/M arrest in Chk1 independent manner, first, we will examine the effect of UCN-01, a potent inhibitor of Chk1 but not Chk2 [207], to see whether it changes the proportion of CDDO-Im-treated SUM159 cells in G2/M. Second, we will perform cell cycle analysis with Chk1 knockdown to confirm whether Chk1 is required for G2/M arrest. We also found that CD44-STAT3 signaling and the NF κ B pathway may be key mechanisms for growth inhibition in SUM159 cells. To prove this hypothesis, first, we will do shorter time point experiments to see whether the regulation occurs in early stage. Second, we will examine other downstream markers of CD44-STAT3 signaling and the NF κ B pathway to find out possible target molecules and knockdown its related genes to prove that molecules regulate the mechanism.

In the second part, we investigated the change of cell population by CDDO-Im in SUM159 monolayers and mammospheres. Next, we would like to analyze both samples by Western blot analysis with cell cycle checkpoint markers, CD44 and its potent downstream signaling markers to find out if there is a difference between monolayers and mammospheres. Also, we plan to investigate if CDDO-Im inhibits the growth of both monolayers and mammospheres by same or different mechanisms.

In vivo studies

We demonstrated that CDDO-Im inhibited the cell growth in both SUM159 monolayers and mammospheres *in vitro*. Next, we plan to investigate the inhibitory effect by CDDO-Im *in vivo*. Cells will divide to different subgroups such as $CD44^{+}/CD24^{+}/ESA^{-}$ or $CD44^{+}/CD24^{-}/ESA^{-}$ from SUM159 cells by cell sorting. Animals would be injected with different subgroups of cells or SUM159 mammospheres into the mammary glands and be treated with different doses of CDDO-Im. Mammary glands and tumors will be analyzed with Western blot analysis and IHC staining. We plan to explore this possibility with *in vivo* studies that CDDO-Im may be a potential agent for TNBC.

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