AMERICAN UNIVERSITY OF BEIRUT

SYNTHESIS OF 7-O-METHYLPUNCTATIN, A NOVEL HOMOISOFLAVONOID, AND ITS MOLECULAR MECHANISMS IN ATTENUATING BASAL AND COX-2-INDUCED MALIGNANT PHENOTYPE OF BREAST CANCER CELLS.

by
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ABSTRACT
OF THE THESIS OF

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Major: Pharmacology and Therapeutics

Title: Synthesis of 7-O-Methylpunctatin, a Novel Homoisoflavonoid, and its Molecular Mechanisms in Attenuating Basal and COX-2-Induced Malignant Phenotype of Breast Cancer Cells

Background: Breast cancer is the leading cause of female morbidity and mortality worldwide. A substantial amount of evidence suggests a major role for inflammation in the initiation and progression of various malignancies including breast cancer. Among the different inflammatory markers, cyclooxygenase-2 (COX-2) is the most investigated and is overexpressed in tumor breast tissue yet undetectable in normal tissue. In fact, phytochemical polyphenols have been reported to decrease the proliferation of cancer cells, block pro-inflammatory cytokines as well as hinder transcription factors that mediate cancer progression.

Methods/Aims: Breast adenocarcinoma cell lines MCF-7 and MDA-MB-231 were employed to study the effects of the synthesized homoisoflavonoid (7-O-methylpunctatin). H^1NMR and C^{13}NMR were done to confirm the structure of our synthesized 7-O-methylpunctatin (7MP). The effects of 7MP were studied on COX-2 overexpressing and basal-breast cancer cells. First, the proliferative (MTT) and migratory (wound-healing assay) capacities of breast cancer cells treated with 7MP were assessed compared to control cells. Following that, prostaglandin E_2 (PGE_2) levels were measured using Enzyme Linked Immunosorbent Assay (ELISA) in COX-2 overexpressing cells. Western blotting was used to evaluate 7MP's selectivity for COX-2 or COX-1. In addition, cell cycle analysis (flow cytometry) and apoptosis markers (caspase-glo assay) were examined on treated cells to evaluate the effect of 7MP on cell cycle and apoptotic machinery. Finally, the phosphorylation level of ERK1/2 (In-cell ELISA) and NF-κB activity (NF-κB luciferase reporter assay) were measured.

Results: The H^1NMR and C^{13}NMR spectra confirmed that our synthesized 7MP has the desired structure as that extracted from *Bellevalia eigii*. 7MP significantly reduced the basal and COX-2-induced proliferation of breast cancer cells. It also reduced the basal migratory capacity of MDA-MB-231 and MCF-7 cells. We also showed that 7MP selectively targets COX-2 but not COX-1 and decreases the amount of released PGE_2 from parent MDA-MB-231 cells and COX-2 overexpressing cells. The cell cycle analysis demonstrated that treatment with 20 μM 7MP caused G0/G1 arrest of breast cancer cells and induced apoptosis by

increasing the activity of caspases 3/7. Finally, 7MP inhibited basal and COX-2 induced ERK1/2 phosphorylation and NF-κB activity in MDA-MB-231 cells.

Conclusion: Our results show that COX-2 potentiates the tumorigenic capacity of breast cancer cells. We also demonstrate that the homoisoflavonoid synthesized 7MP attenuates both basal and inflammation-potentiated malignant phenotype of breast cancer cells.

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CHAPTER I

INTRODUCTION

A. Background information

1. Epidemiology

Breast cancer is one of the most common cancers [1]. In 2016, 1.7 million new breast cancer cases arose making it the leading cause of female morbidity and mortality worldwide [2]. Resulting in a contribution of 25.4% of all cancer cases and 15% of deaths by cancer in women globally in 2018 [3]. Lebanon has its share of these numbers since breast cancer rates in this small developing country are amidst the highest in the region and worldwide [4]. It has been reported that countries with higher socio-economic achievement, also known as higher human development index (HDI), have higher incidence of breast cancer as opposed to countries with low or medium HDI [5]. On the other hand, deaths due to breast cancer over the period of 1990-2016 were higher in countries with low HDI, in part due to the lack in accessibility to therapy and diagnosis [6]. Having said that, it is expected that breast cancer incidence will continue to escalate in the future subjecting both developed and developing countries to multifaceted challenges [6].

2. Risk Factors

There are various health, environmental, and reproductive risk factors that are correlated with the development of breast cancer [7]. Many crucial risk factors in breast cancer are non-

modifiable, such as age and hereditary mutations. Others, on the other hand, such as those related to the lifestyle of individual, have been found to be potentially modifiable (diet, exercise and smoking) [8].

a. Age and Hereditary factors

The incidence of breast cancer is strongly correlated to aging. According to the cancer statistics in 2017, 99.3% of breast cancer related deaths in America were of women aged above 40, and around 71.2% were for those aged above 60 [9]. Besides aging, family history is also an important indicator of breast cancer risk. A cohort study of more than 113,000 women established that having first-degree relatives with breast cancer increases the risk of developing the disease to 2.5 folds [10]. In addition, inherited mutations the tumor suppressor genes: BRCA1 and BRCA2 are partially accountable for the inherited susceptibility for the disease [11]. Women with such mutations will more probably develop breast cancer by the age of 70 compared to non-carriers [11].

b. Reproductive Factors

There are multiple reproductive factors involved in the increased risk of breast cancer. It has been shown that each 1-year delay in menopause increases the risk of breast cancer by 3% whereas a similar delay in menarche would decrease the risk by 5% [12]. In brief, the increased time span in which a woman is exposed to sex hormones, the higher the risk of her developing breast cancer [13].

c. Lifestyle (Alcohol consumption, smoking, fitness and nutrition)

There are several lifestyle related risk factors in breast cancer but only three will be in this paper. The first being alcohol consumption, in which several studies suggested that it possesses a dose dependent relationship with increased breast cancer risk. The underlying mechanism is most likely related to the fact that alcohol consumption results in an increase of estrogen levels in blood since the breast cancers associated with alcohol are of the ER (+) subtype.

There are conflicting data on the effect of smoking on the incidence of breast cancer. Some studies have shown that women who smoke before their first pregnancy or are heavy smokers have a relatively greater risk of developing breast cancer. On the other hand, smoking after menopause has been associated with decreased risk of breast cancer. The latter could be due to tobacco's anti-estrogenic effects.

Physical exercise and the high consumption of fruits and vegetables have been linked to the decrease in breast cancer risk. Moreover, increased adipose tissue in women transitioning into menopause increased breast cancer risk by 12% due to the increase of estrogens in blood [14].

d. Estrogen

Estrogens can be divided into two main categories: endogenous (produced by the ovaries) or exogenous (synthetic or phytoestrogens). Both exogenous and endogenous estrogens have been shown to be involved in the increased risk of breast cancer. Furthermore, ovariectomy has been associated with the decreased risk of breast cancer [15]. Hormonal replacement therapy or the administration of oral contraceptives have been also risk factors in breast cancer [15].

Hormonal replacement therapy (HRT) is the administration of exogenous estrogens alongside other hormones to menopausal or post-menopausal women in order to compensate the reduced levels of the endogenous ones [16]. After publishing on the adverse effects of HRT in 2003 and the subsequent reduction in its use, the incidence rate of breast cancer in America decreased by 7% [17].

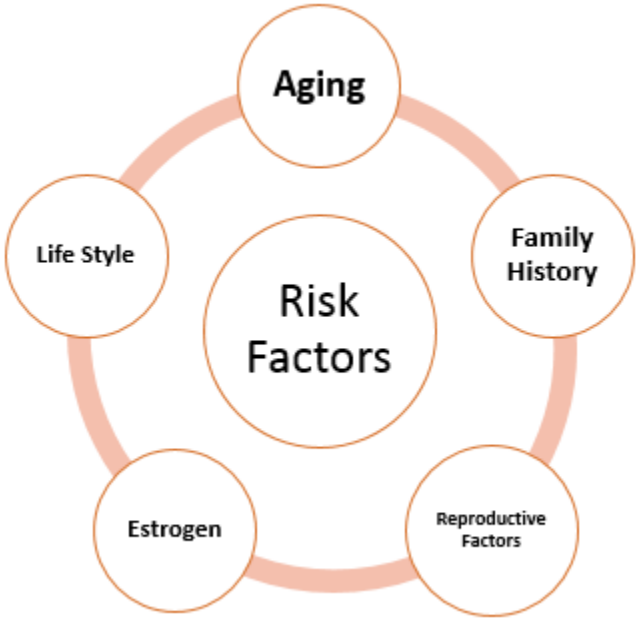

Figure 1 Risk factors associated with Breast Cancer.

B. Screening

Screening is crucial for an early detection of breast cancer [18]. Different types of studies such as case control studies, observational studies and RCTs have shown that mortality due to breast cancer decreased after screening mammograms [19]. A mammogram enables the visualization of the internal structures of the breasts with a relatively high sensitivity and specificity [20]. Dense breasts can reduce the sensitivity of mammography screening

therefore, in such cases adjunctive screening is recommended such as digital breast tomosynthesis (DBT), breast MRI, or ultrasound.

DBT uses several X-rays to construct a 3D-image of the breasts for a clear display of breast tissue [19]. MRI, on the other hand uses magnetic fields and this would construct a detailed cross-sectional image of the body. MRI has a higher sensitivity than mammography screening and is used in conjunction with it in high risk women [19]. If the woman is unable to undergo MRI due to creatinine clearance issues, an ultrasound is carried out instead.

C. Subtypes & Therapy

Breast cancer is a heterogenous disease [21]. It is histologically classified into three major subtypes based on the different molecular markers the breast tumors are expressing (estrogen receptor [ER], progesterone receptor [PR], human epidermal growth factor receptor 2 [ERBB2/HER2]) [22]. In 2000, Perou et al further stratified breast cancer into more than four "intrinsic subtypes" based on gene expression patterns of human-breast tumor specimens [23]. Basal like and HER-2 positive are two subtypes that are derived from ER-negative tumors while luminal A and luminal B subtypes are derived from ER-positive tumors [24, 25]. The basal-like category is mainly comprised of triple negative breast cancers (TNBCs) i.e. those lacking ER, PR, HER2 receptors [26]. TNBCs are highly invasive and are associated with shorter relapse-free survival period [23].

Most breast cancer patients are of the hormonal receptor positive subtype (70% of patients), the rest of the patients are distributed among TNBCs (15%) and ERBB2/HER2 positive subtypes (15%-20%) [27]. Breast cancer is divided into four stages I-IV, with IV being the most severe in which the tumor has already metastasized into distant organs [28]. Stage I on the other

hand, is when the tumor size is small (<2 cm) with no involvement of the lymph nodes. The median survival time among all stages is the least for TNBCs relative to HR (+) or ERBB2/HER2(+) [29-31].

The standard approach for treating breast cancer is breast-conserving surgery and radiation in addition to other treatment options such as chemotherapy and endocrine therapy [32]. The classification of breast cancer into subtypes helped specialize the treatment for each subtype of the disease in a unique manner. “Trastuzumab” is a therapeutic agent used for breast cancers which over-express the ERBB2 receptor, by virtue of its ability to target this receptor and consequently inhibiting its downstream signaling [33]. On the other hand, for those expressing hormonal receptors, endocrine therapy is preferred by using agents such as tamoxifen or fulvestrant [32]. Targeted therapy has somehow helped with the improvement of the clinical outcome of the various subtypes of breast cancer except that of TNBC, which has been rendered refractory to such therapies [34, 35]. Multiple problems are associated with the conventional treatment of breast cancer such as the side effects of chemotherapy and radiotherapy in addition to multidrug resistance [36-39]. This inconvenience associated with the conventional treatment has sparked interest in searching for alternative agents that can be used as adjuvants to existing treatments or as chemotherapeutic agents on their own [40-43].

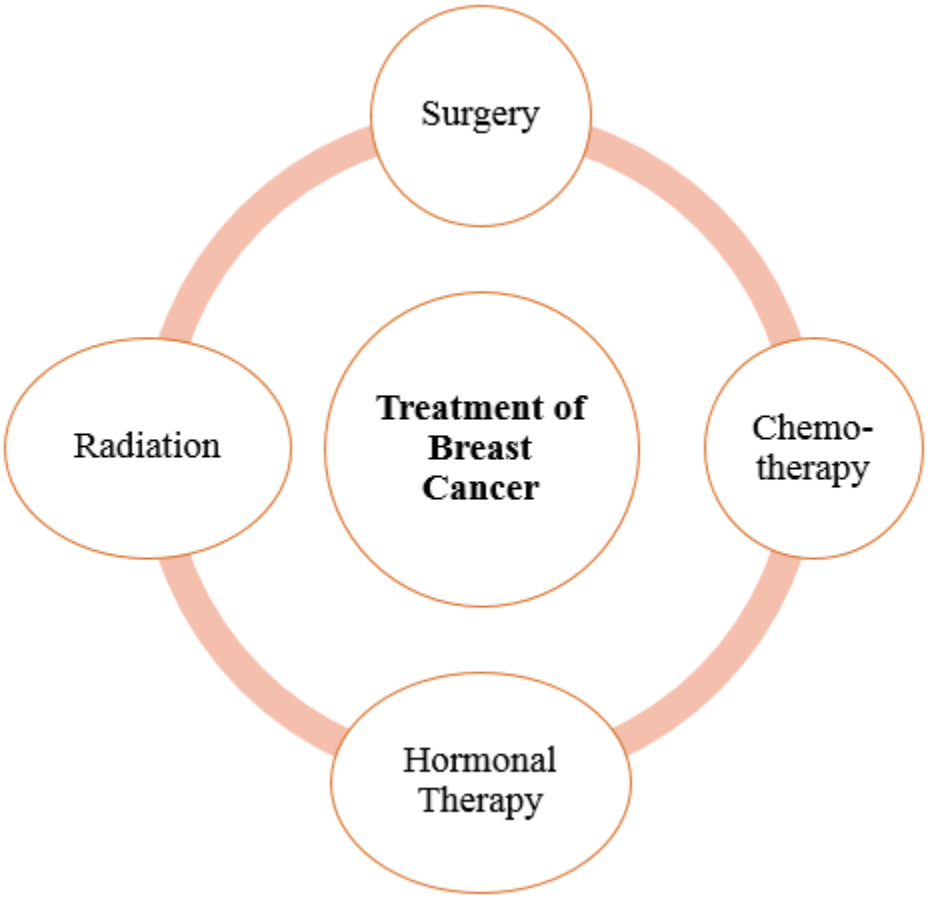

Figure 2 Breast cancer therapeutic options.

1. Natural products in treatment of breast cancer

In the field of cancer, numerous natural products have displayed beneficial effects with less toxicity compared to synthetic compounds [44, 45]. Over the last four decades, more than 60% of the approved small molecules used in anti-cancer therapy are natural products or derived therefrom [46]. A class of plant-based polyphenolic compounds, named "flavonoids", that is abundant in fruits and vegetables have been associated with the treatment and prevention of different ailments including cancer [47-51]. The family of flavonoids is comprised of different subclasses one of which is the family of 3-benzylidenechroman-4-ones termed "Homoisoflavonoids" [52, 53].

a. Homoisoflavonoids

The term “homoisoflavonoids” was first coined in 1967 to describe a group of natural compounds that were similar to isoflavonoids but had an extra carbon in their skeleton [54]. Homoisoflavonoids are rarely found in nature and can be further divided into five groups based on their structures: sappanin-type, protosappanin-type, scillascillin-type, brazilin-type, and caesalpin-type [55]. Most of the isolated homoisoflavonoids originate from the two plant families Asparagaceae and Fabaceae; the rest however, are divided among other families such as the Polygonaceae, Portulacaceae, Orchidaceae, and the Liliaceae [55].

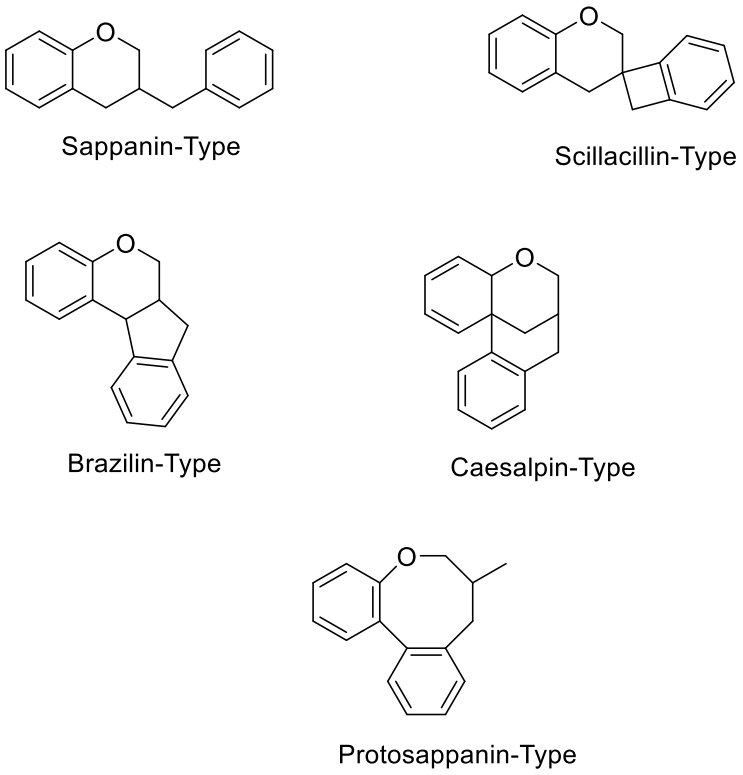

Figure 3 Molecular scaffolds of the different types of homoisoflavonoids described in the literature.

Although it has been around five decades since the first homoisoflavonoids were isolated, more is known about their chemistry compared to their bioactivity [56]. A wide range of biological activities have been reported by these compounds including: antioxidant [57], anti-mutagenic [58], anti-inflammatory[59], and anti-cancerous [60]. Although there aren't extensive studies on the direct effects of homoisoflavonoids in breast cancer models (in vivo and in vitro); many homoisoflavonoids did show desired effects on models of other cancer types. Homoisoflavonoids have been shown to modulate different signaling pathways in cancer that can be hypothetically applied to breast cancer.

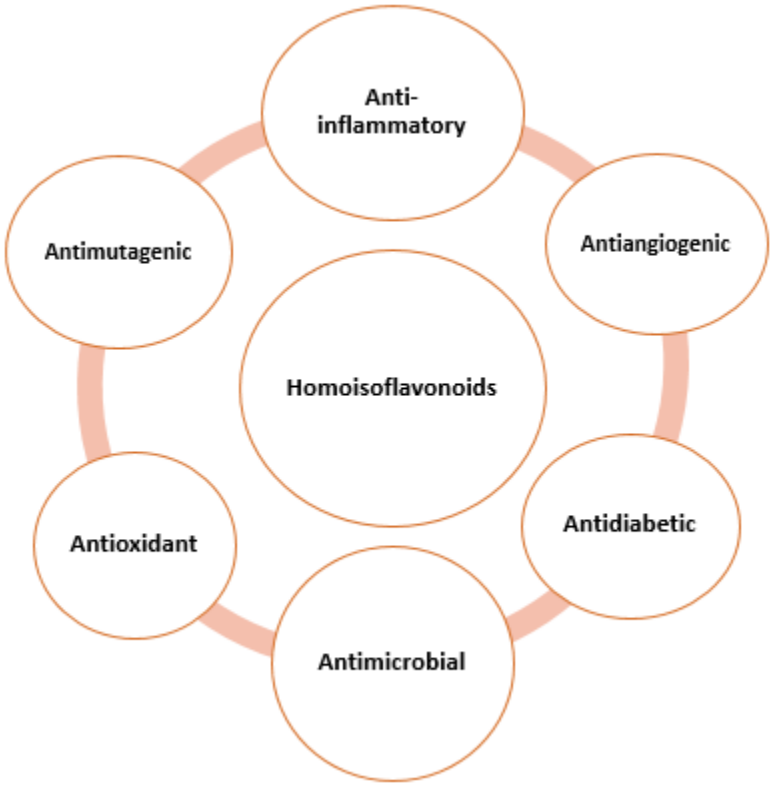

Figure 4 Reported biological activities of Homoisoflavonoids.

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D. Inflammation in Breast Cancer

Inflammation is one of the defense mechanisms the body utilizes upon infection, injury or exposure to irritants [61]. Chronic inflammation that persists for a long period of time creates an environment that can lead to and is suitable for different ailments including cancer [62] [63]. Inflammatory breast cancer (IBC) is one of the most aggressive forms of the disease and it represents around 2-10% of breast cancer tumors [64, 65]. Moreover, inflammatory microenvironment is associated with therapeutic resistance in addition to promoting angiogenesis, metastasis and an immunosuppressive environment [66]. One of the major dysregulated inflammatory mediators in cancer is COX-2 and is often linked to the increased rate of death by cancer.

1. The COX-2/PGE2/EP axis

Cyclooxygenases are enzymes that catalyze the formation of prostaglandins from arachidonic acid (AA). They exist in two distinct forms: COX-1 and COX-2. COX-1 is constitutively expressed in almost all body tissues while COX-2 expression is induced upon inflammation [67]. A huge body of evidence indicates that COX-2 is an important player in the cross talk between inflammation and cancer development [68-70]. In fact, the overexpression of COX-2 is a prognostic marker of stage III breast cancer and is associated with shorter relapse-free survival period [68]. Moreover, COX-2 have been considered the major source of prostaglandins; which are key players in inflammation [71]. The family of prostaglandins includes PGE_2, PGF_2, TXB_2, thromboxane A_2 (TXA_2), PGD_2, and PGI_2.

The elevated levels of PGE_2 have been associated with various malignancies and decreased survival rates [72]. PGE_2 can cause cancer progression by promoting cancer cell

proliferation, migration, invasion, and growth [73, 74]. Prostaglandin uptake transporter (PGT) and multiple drug resistance associated protein (MRP4) are two transporters that carry PGE_2 in and out of the cell respectively [75, 76]. These two transporters are therefore able to control the extracellular levels of PGE_2 and subsequently affecting cancer development [77]. PGE_2 exported out of the cells can bind to EP receptors which are G-protein coupled receptors.

Upon activating the EP receptors, PGE_2 can transactivate multiple signaling pathways such as nuclear factor-kappa B (NF-κB) [78], mitogen-activated protein kinase (MAPK) [79], phosphoinositide 3 kinase-protein kinase B (PI3K-PKB/ Akt) [73], and hypoxia inducible factor (HIF)-1α-associated pathways [80]. In addition, PGE_2 activation of EPs can also crosstalk with pro-tumorigenic extracellular signaling-regulated kinase (ERK)/c-Jun N-terminal kinases (JNKs) [81, 82].

2. EP receptors in Breast Cancer

There are four subfamilies of EP receptors (EP1-4) each with distinct biochemical properties and biological outcomes [83]. EP1 causes an increase in intracellular calcium levels through coupling with Gαq or through receptor-activated calcium channels (RACCs) [84, 85]. EP3 receptors exist in different isoforms but they can either inhibit adenylyl cyclase (coupled to Gαi) or increase IP3/$[Ca^{2+}]_i$ [86-88]. On the other hand, EP2 and EP4 are coupled to Gαs and adenylyl cyclase and can in turn stimulate the second messenger cAMP along with its downstream effector: protein kinase A (PKA) [89-91].

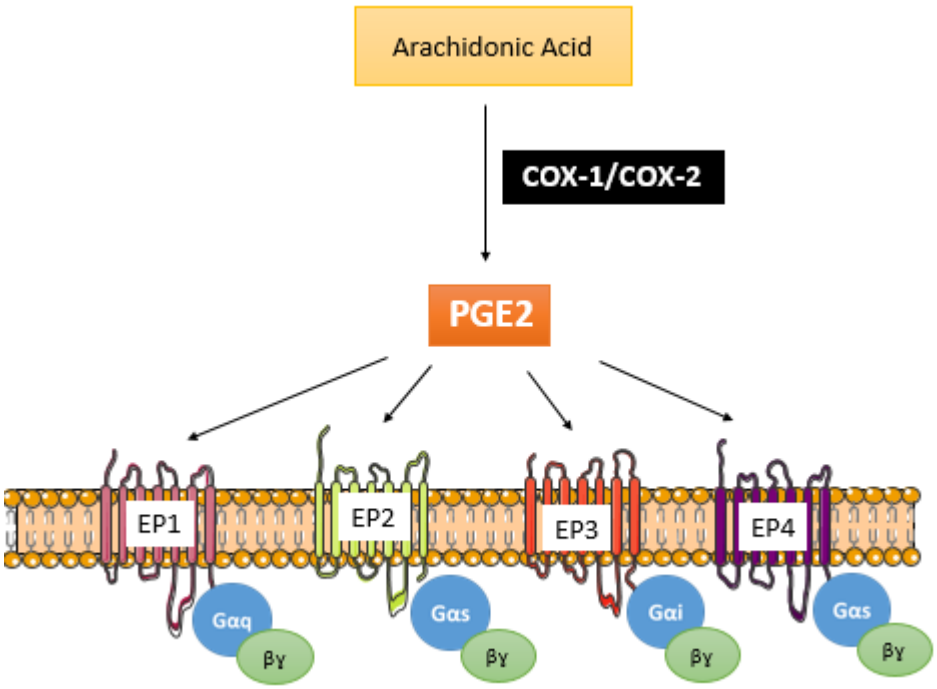

Figure 5 The different couplings for the G-protein coupled receptors (EP1-4) to which PGE2 bind to.

Different EP receptors are induced in the different stages of normal murine mammary gland development [92]. Both EP1 and EP3 receptors are expressed during the involution phase of the gland meanwhile EP3 receptors can also be found scarcely in the proliferative phase [92]. On the other hand, EP2 and EP4 receptors are only induced in the proliferative phase of the development of the gland [92].

In mammary tumorigenesis, the expression pattern of EP receptors changes. The expression of EP1, EP2 and EP4 receptors increases yet that of EP3 is downregulated. In a model of mammary tumorigenesis, the expression of both EP2 and EP4 receptors was suppressed after the treatment with a non-selective COX-1 and COX-2 inhibitor. Moreover, studies have suggested that EP2 and EP4 play a pivotal role in the progression and angiogenesis of COX-2-induced mammary tumors [92]. EP2 and EP4 are also involved in the PGE_2-mediated increase of aromatase activity in breast cancer cells [93]. Aromatase catalyzes the conversion

of androgens to estrogens and such an increase in its activity would promote the growth of estrogen-dependent tumors [94].

Huge evidence suggests that among the four receptors, targeting EP4 seems to be beneficial in breast cancer therapy. A study showed that using an agonist of EP4 increased the proliferation and migration of SUM149 cells (inflammatory breast cancer cell line) while blocking EP4 had an anti-proliferative and anti-migratory effect [95]. As a matter of fact, numerous studies have shown the involvement of EP4 in different cancers such as lung, prostate, and colon cancer [96-98]. In breast cancer models, EP4 have also been implicated in modulating many aspects of metastasis including migration and invasion [99, 100].

3. COX-2/PGE2/EP axis in Breast Cancer

Huge experimental evidence suggests that COX-2 over expression alone can induce breast cancer in animal models [101-103]. Breast tumors overexpressing COX-2 are large in size and highly invasive [104]. Likewise, gene expression analyses in mouse models have associated COX-2 with the ability of breast cancer to metastasize to the bones, brain and lungs [59, 105]. Remarkably, silencing COX-2 prevents the migration of breast cancer cells *in vitro* [106]. In addition, a study showed that breast cancer patients who responded to chemotherapy shifted from a COX-2 positive to negative expression profile; with an improvement in their progression-free survival [107].

4. COX-2/PGE2/EP axis and EMT progression

Epithelial to mesenchymal transition (EMT) is a process involved in multiple pathological conditions including cancer cell metastasis and resistance to therapy [108-110]. In EMT, epithelial cells lose their polarity and cell-cell adhesion and become of a mesenchymal phenotype [111]. The process of EMT is characterized by the loss of epithelial markers such as E-cadherin and β-catenin and increase in the expression of mesenchymal markers such as vimentin and N-cadherin [112]. The acquisition of a mesenchymal phenotype ultimately leads to the intravasation of cancer cells. A previous study showed that COX-2/PGE_2 downregulate the expression of cell adhesion molecule E-cadherin, and therefore promote EMT [113]. Moreover, COX-2 and PGE_2 are associated with the increased expression of various mesenchymal markers such as α-SMA, vimentin, and zinc finger protein SNAI1. In addition, knocking down COX-2 using siRNA or the use of an EP4 receptor antagonist reversed the TAM-induced EMT in an in vitro model of lung cancer [114].

E. Homoisoflavonoids and Inflammation

Macrophages are immune cells that are involved in the modulation of inflammatory responses by virtue of their ability to secrete a variety of inflammatory mediators (PGE_2, IL-6, TNF-α, NO and others) [115]. A major player in the pathogenesis of inflammation is the small diffusible molecule: nitric oxide (NO). The sappanin-type homoisoflavonoids, portulacanones A-D, have been reported to decrease the production of NO from LPS-induced RAW-264.7 macrophages [116]. This decrease in NO production is due to the decrease in the expression of the inducible nitric oxide synthase (iNOS) [116]. Another sappanin-type homoisoflavonoid, 4'-O-demethylophiopogonanone E, extracted from the rhizome of *Ophiopogon japonicus* significantly inhibited the release of NO and pro-inflammatory cytokines IL-1β and IL-6 at non-

cytotoxic concentrations (< 50 µM) [117]. Also, the remarkably high homoisoflavonoid content (dihydroeucomin and eucomol) of *Agave pygmaea* have rendered it one of the strongest anti-inflammatory extracts among the Agave taxon [118].

Upon injury or inflammation, antioxidant proteins are utilized to protect the body against oxidative damage [117]. Among the family of transcription factors that modulate the expression of anti-oxidant proteins is the nuclear factor erythroid 2-related factor 2 (Nrf2) [117]. A homoisoflavonoid from the heartwood of *Caesalpinia sappan*, Sappanone A, has been shown to increase the translocation of Nrf-2 to the nucleus and subsequently increasing the expression of Nrf-2 target genes such as heme oxygenase-1 (HO-1) [119]. HO-1 plays a key protective role in tissues by reducing inflammatory responses and maintaining cellular homeostasis [120-122]. Not only did Sappanone A act on the Nrf-2 pathway, it also suppressed the LPS-induced activation of NFκB in RAW-264.7 macrophages [119].

Major agents in inflammation, cyclooxygenases, are enzymes that catalyze the formation of eicosanoids from arachidonic acid [123]. The over-expression of COX-2 in human breast cancer has been associated with poor prognosis, increased proliferation, and metastasis to lymph nodes [104, 124, 125]. Furthermore, the production of PGE_2 through COX-2 in breast tumors have been shown to dampen the activity of the immune system, and it is now evident that blocking COX-2 could reverse this problem [126]. Although non-selective cyclooxygenase inhibitors attenuate inflammatory responses, they are associated with gastro-intestinal side effects [127]. Nevertheless, it has been demonstrated that the selective inhibition of COX-2 attenuates inflammation and exerts cancer preventative effects, concomitant with less toxicity compared to non-selective cyclooxygenase inhibitors [128-130]. A study demonstrated that three homoisoflavonoids of the Scillascillin-type: (R)-3-(3’,4’-

Dihydroxybenzyl)-7-hydroxy-5-methoxychroman-4-one,(E)-3-(3',4'-dihydroxybenzylidene)-7-hydroxy-5-methoxychroman-4-one, and ovatifolionone acetate were shown to have significant inhibitory activity against COX-2 [131].

F. Homoisoflavonoids and Other hallmarks of Cancer

1. Proliferation

Proliferation is a major facet that causes tumor progression and development [132]. By disrupting multiple signaling pathways, cancer cells maintain a very high proliferation rate [133]. In cancer therapy, it is favorable to target this markedly fast proliferation and uncontrolled division. For this purpose, some homoisoflavonoids were tested against breast cancer cell lines *in-vitro*. Interestingly, homoisoflavonoids extracted from "*Bellevalia flexuosa*", have shown growth inhibitory effects against the estrogen receptor negative breast cancer cell line MDA-MB-231 [134]. In addition, a rare scillascillin-type homoisoflavonoid from the seeds of *Crotalaria pallida,* had anti-proliferative effects against MCF-7 cells with an IC50 of 6.77 μM [135].

2. Angiogenesis

Angiogenesis is the sprouting of blood vessels from pre-existing ones; a process that is crucial for the progression of tumors [136]. It is an essential step for the local growth of tumors leading to metastasis, with vascular endothelial growth factor (VEGF) being a key player in this process [137]. Under hypoxic conditions, hypoxia-inducible factor (HIF)-1α is stabilized and in turn enhances the transcription of hypoxia-inducible genes such as that of VEGF. It has been demonstrated that targeting VEGF expression, reduces tumor progression by aberrating the

process of angiogenesis. In fact, homoisoflavonoids extracted from *Ophiopogon ohwii*, significantly inhibited the activity of HIF-1α in HepG2 cells [138]. These same homoisoflavonoids were able to inhibit the migration and tube formation of human umbilical vein endothelial cells (HUVECs) at a very low concentration (1 μM) [138].

There are several drugs that aim to inhibit angiogenesis via targeting VEGF such as Avastin (bevacizumab); however, they are associated with side effects and resistance [139]. Finding a molecule that could target VEGF and henceforth be used on its own or as adjuvants to existing anti-VEGF molecules is favorable in the treatment of breast cancer. A sappanin-type homoisoflavonoid from the bulbs of *Cremastra appendiculate*, namely cremastranone, was able to inhibit tube formation, migration, and (bFGF)-induced invasion of HUVECs in-vitro [140]. Moreover, a synthetic derivative of cremastranone "SH-11037" has been developed and showed synergism alongside other anti-VEGF treatments in *in-vivo* and *in-vitro* models of ocular neovascularization [141].

3. Invasion and Migration

The extracellular matrix (ECM) is a scaffold composed of a variety of components including laminins, elastin, fibronectin, collagen and other glycoproteins [142]. The degradation of the ECM by matrix metalloproteinases (MMPs) has been associated with the invasion and metastasis of cancer cells for over than four decades now [143]. The role of MMPs in tumorigenesis isn't solely dependent on their ability to remodel the ECM and influence cell migration; but also, on their ability to modulate other cell signaling pathways related to cell growth, inflammation and angiogenesis [144]. It has been demonstrated, that the use of an inhibitor of MMPs in combination with a HER-2/neu kinase inhibitor synergistically impedes

the growth of breast cancer xenografts [145]. A study showed that the treatment of MCF-7/HER-2 neu cells with the homoisoflavonoid "brazilin" causes the downregulation of matrix metalloproteinases MMP-2 and MMP-9 as well as the expression of HER-2 receptor [146].

G. A Novel Homoisoflavonoid: 7-O-Methylpunctatin

As mentioned earlier, homoisoflavonoids constitute a rare class of natural compounds that are interesting due to their wide array of biological effects [55]. 7-O-Methylpunctatin (7MP) is a sappanin-type homoisoflavonoid extracted and purified from *Bellevalia eigii* which is a plant that belongs to the Asparagaceae family [147].

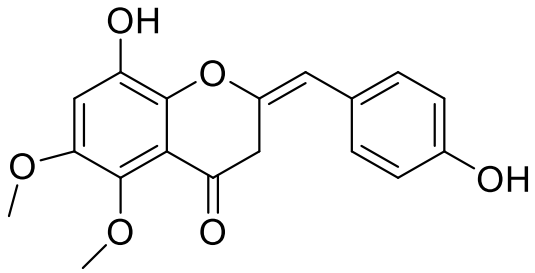

Figure 6 Chemical structure of 7-O-Methylpunctatin

Our laboratory has recently published on the effects of 7MP on the phenotypic switch of human arteriolar smooth muscle cells [148]. The switch to a synthetic phenotype is characterized by an increased invasion, migration, and proliferation of formerly contractile vascular smooth muscle cells (VSMCs) [149]. In the published study, 7MP decreased FBS-induced cell proliferation, migration, invasion, and adhesion of microVSMCs [148]. Remarkably, 7MP was also able to abolish FBS-induced expression of MMP-2, MMP-9, and NFκB, as well as reducing the activation of ERK1/2 [148].

The depicted 7MP effects suggest that it may amend arteriolar inflammation and possibly considered cardio-protective [148]. Since inflammation is now well-recognized to be a major player in the onset and progression of many tumors including breast cancer, this renders 7MP a potential modulator of signaling pathways in breast cancer.

H. Rationale and Hypothesis

The search for a more effective therapeutic regimen for breast cancer is a prime goal. And since inflammation mediated by COX-2 have been intricately linked to breast cancer malignancy, finding a molecule that could inhibit inflammation and hallmarks of breast cancer is a tempting option. Interestingly, homoisoflavonoids, a family of phytochemical polyphenols, have been reported to possess anti-inflammatory and anti-cancer activities. Moreover, 7MP belongs to the family of homoisoflavonoids and is originally found in nature. We therefore aim to:

1. To synthesize and characterize the homoisoflavonoid 7MP.
2. Assess the ability of 7-O-methylpunctatin to attenuate the basal and COX-2-induced malignant phenotype of breast cancer cells.

We hypothesize that 7MP can attenuate the inflammation-potentiated malignant phenotype of breast cancer cells by targeting COX-2 or COX-2 mediated pathways.

CHAPTER II

MATERIALS & METHODS

A. Materials used in the synthesis:

Benzene-1,3,5-triol, acylating agent, aluminum chloride, benzyl chloride, hexamethylphosphoramide (HMPA), dichloromethane, methanol, ethanol, ethyl formate, sodium hydride, hydrochloric acid, Lindlar's Catalyst, cellite, methyl iodide, potassium carbonate, potassium persulfate, potassium hydroxide, silica.

B. Materials used in biological assays:

1. Cell Culture:

The breast adenocarcinoma cell lines MCF-7 and MDA-MB-231 were both cultured in *Dulbecco's Modified Eagle's medium* – High Glucose (DMEM-HG) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown in 10 mm dishes and maintained in a humidified incubator at 37°C with 5% CO_2 atmosphere. Culture medium was changed every 48 hours and cells were split in a ratio of 1 to 4 once reaching 90% confluence. Culture media and FBS were purchased from Sigma-Aldrich. Penicillin/streptomycin was obtained from Lonza.

2. Wound healing or scratch Assay:

MCF-7 and MDA-MB-231 cells were seeded in a 12-wells plate ($6x10^5$ cells/well) in 1 ml of DMEM-HG. When the cells reached around 90% confluency, a scratch was created manually

through the confluent monolayer using a yellow P10 tip (2–20 μl). The culture medium was then aspirated, and wells were washed with PBS to remove cellular debris. Complete fresh medium was then added.

The control wells were treated with 0.1% DMSO and the treatment condition of the other wells was 20 μM of 7MP.

Photomicrographs were taken at baseline (0 hour) and consecutively every two hours until 12 hours. ZEN lite from Zeiss Microscope software was used to measure the width of the scratch which reflected the extent of cell migration. The distance migrated was measured in µm.

3. Viability Assay:

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. MTT was obtained from abcam (ab146345). MCF-7 and MDA-MB-231 cells were seeded in 96-well plates (6000 cells/well) in 200 µl complete DMEM. 24 hours later, media were aspirated, and fresh medium was added. Vehicle treated cells (0.1% DMSO) represented the control group. Cells were treated with increasing concentrations of 7MP (10, 20, 40, 60, 80, 100 µM) at three different time points (24, 48 and 72 hours). 20 µl MTT (5mg/ml) was then added to each well and cells were incubated at 37 ^{o}C for 3 hours. All culture media were then removed, and the formazan violet crystals were dissolved in 200 µl DMSO. Treatments were performed in duplicates. Cell viability was assessed colorimetrically at a wavelength of 595 nm using Elisa Multiscan EX Reader (Thermo), where the intensity of violet color is directly proportional to the number of viable cells.

4. Transient Transfections:

Cells were transiently transfected with COX-2 plasmid using Lipofectamine 2000, to achieve an approximate 80% transfection efficiency. Cells were then allowed to recover overnight in complete growth medium.

5. Western blotting:

Cells were lysed using 10 mM Tris pH 6.8, 2% SDS. Proteins were quantified by DC protein assay kit (Bio-Rad, USA). Protein samples were then loaded (30-50µg) on 5-11% SDS-PAGE along with protein ladder (Abcam), electrophoresed at 70 V (Bio-Rad, USA) and then transferred to PVDF membranes (Bio-Rad, USA). After blocking with 5% fat free dry milk in TBS-T (TBS and 0.05% Tween 20) for 1 hour at room temperature, the blots were probed with primary antibody at 4 ^{o}C overnight. Blots were then washed three times with TBS-T and incubated with Horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:2000) for 1 hour at room temperature. Immunoreactive bands were finally detected by ECL chemiluminescent substrate (Bio-Rad, USA). Blot images were taken by using Chemidoc MP Imaging system.

6. In-cell ELISA

Cells are seeded in 96-well plate. After the adherence of cells, they are treated with 7MP (20µM) or with DMSO as for control. After 1 hour of treatment, fix the cells with 8% paraformaldehyde and let them incubate for 15 minutes. Aspirate the wells then wash with 1X PBS. Add 200µl of 1X permeabilization buffer to each well and incubate for 30 minutes.

Aspirate the permeabilization buffer then add blocking buffer and incubate for 2 hours. Add primary antibody to each well and incubate for overnight at 4^{o}C. Remove primary antibody then wash 3X using 1X wash buffer. Remove wash buffer then add diluted secondary antibody (HRP conjugated) and incubate for 2 hours. Remove secondary antibody then wash wells with 1X wash buffer. After that, add 100 µl/well of HRP substrate then read using a colorimeter.

7. NF-κB luciferase Rerpoter

Cells were transiently transfected with NF-κB-driven promoter luciferase using Amaxa Nucleofector (Amaxa Biosystems, Gaithersburg, MD, USA). Renilla luciferase vector was used as an internal control, to which firefly luciferase values were normalized. Following transfection, cells were left to recover overnight, then treated with 7MP (20µM) or DMSO for control. After 24 hours, luciferin is added to the wells luminescence is measured by a luminometer.

8. Analysis of PGE_2 Production

PGE_2 levels were measured using the PGE_2 Enzyme linked immunosorbent assay (ELISA) kit (R&D Systems) according to manufacturer's protocol.

9. Cell Cycle Analysis

Cells were treated for 48 h with complete medium in the absence or presence of 7MP. After washing the cells by PBS, cells were trypsinized then collected by centrifugation. Collected

cells were then washed twice with cold PBS then resuspended in 500 µL of PBS. The cells were then fixed by 2 mL of ice-cold pure ethanol for 15 minutes. This suspension was centrifuged again, and the pellet was washed with PBS. Cells were then incubated for 10 min in 1 mg/mL of propidium iodide in PBS. Propidium iodide (PI) fluorescence was read using Guava EasyCyte8 Flow Cytometer (Luminex, Hayward, CA, USA). Cell cycle analysis was done using Guava Soft 2.7 software.

10. Caspase-Glo assay

The 96-well plates containing treated cells are removed from the incubator and allowed to equilibrate to room temperature. 100µl of Caspase-Glo 3/7 reagent is added to each well containing control cells or treated cells in culture medium. The plate is then sealed. The contents of wells are then mixed gently using a shaker at 300 rpm for 30 seconds. Incubate at room temperature for 1 hour. Finally, the luminescence of each sample is measured in a plate-reading luminometer.

11. Chorioallantoic membrane (CAM) assay

Fertilized eggs were put in a 37^{o}C incubator humidified (50%) with rotation. After a week of incubation, a small window is created using sharp metal edge above the CAM. After this window was made, 30 µL of 20 µM of 7MP prepared in PBS was added above the CAM and for the control the same amount with DMSO in PBS was used. The window was then sealed with parafilm and the egg was returned to the same incubator. Images were taken after 24 hrs and analyzed using AngioTool.

12. Statistical Analysis

Statistical analysis was performed by a student's t-test for either paired or unpaired observations using GraphPad Prism. Data are presented as mean ± SEM, where n is equal to the number of times an experiment is repeated (n will be = 3). When more than two means are used for comparison, ANOVA was used: either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test). $p < 0.05$ is considered significant.

CHAPTER III

RESULTS

A. Synthesis of 7-O-Methylpunctatin

The synthesis of 7MP starts with the acylation of benzene-1,3,5-triol **(1)** yielding 1-(2,4,6-trihydroxyphenyl)ethan-1-one **(2)**. Benzyl chloride was then added to **(2)** alongside K_2CO_3 as a base and Hexamethylphosphoramide (HMPA) as a solvent; a reaction that gives off 1-(2,4-bis(benzyloxy)-6-hydroxyphenyl)ethan-1-one **(3)** as a product. **(3)** is then dissolved in ethyl formate at -10 ^{o}C then sodium hydride (NaH) is added to the mixture and kept stirring. After 4 hours of stirring, methanol (MeOH) and hydrochloric acid (HCl) are added to the reaction mixture consecutively to obtain 5,7-bis(benzyloxy)-4H-chromen-4-one **(4). (4)** is then hydrogenated using Palladium over carbon as a catalyst yielding 5,7-dihydroxychroman-4-one **(5).** A two-step methylation reaction is then performed by deprotonating the hydroxyl group of **(5)** using K_2CO_3 followed by the addition of a methyl group using methyl iodide. An Elbs persulfate oxidation reaction is then performed on **(6)** using $K_2S_2O_8$ and KOH which then gives 5,8-dihydroxy-7-methoxychroman-4-one **(7).** A second methylation reaction is then performed on **(7)** that produced 5-hydroxy-7,8-dimethoxychroman-4-one **(8).** A protected benzaldehyde namely 4-(methoxymethoxy)benzaldehyde is then left to react with **(8)** in a solution of ethanol and KOH overnight producing (E)-5-hydroxy-7,8-dimethoxy-3-(4-(methoxymethoxy)benzylidene)chroman-4-one **(9)**. Methanol and diluted HCl are then used to deprotect **(9),** a reaction that lead to our final product which is (E)-5-hydroxy-3-(4-hydroxybenzylidene)-7,8-dimethoxychroman-4-one **(10)** also known as 7-O-methylpunctatin.

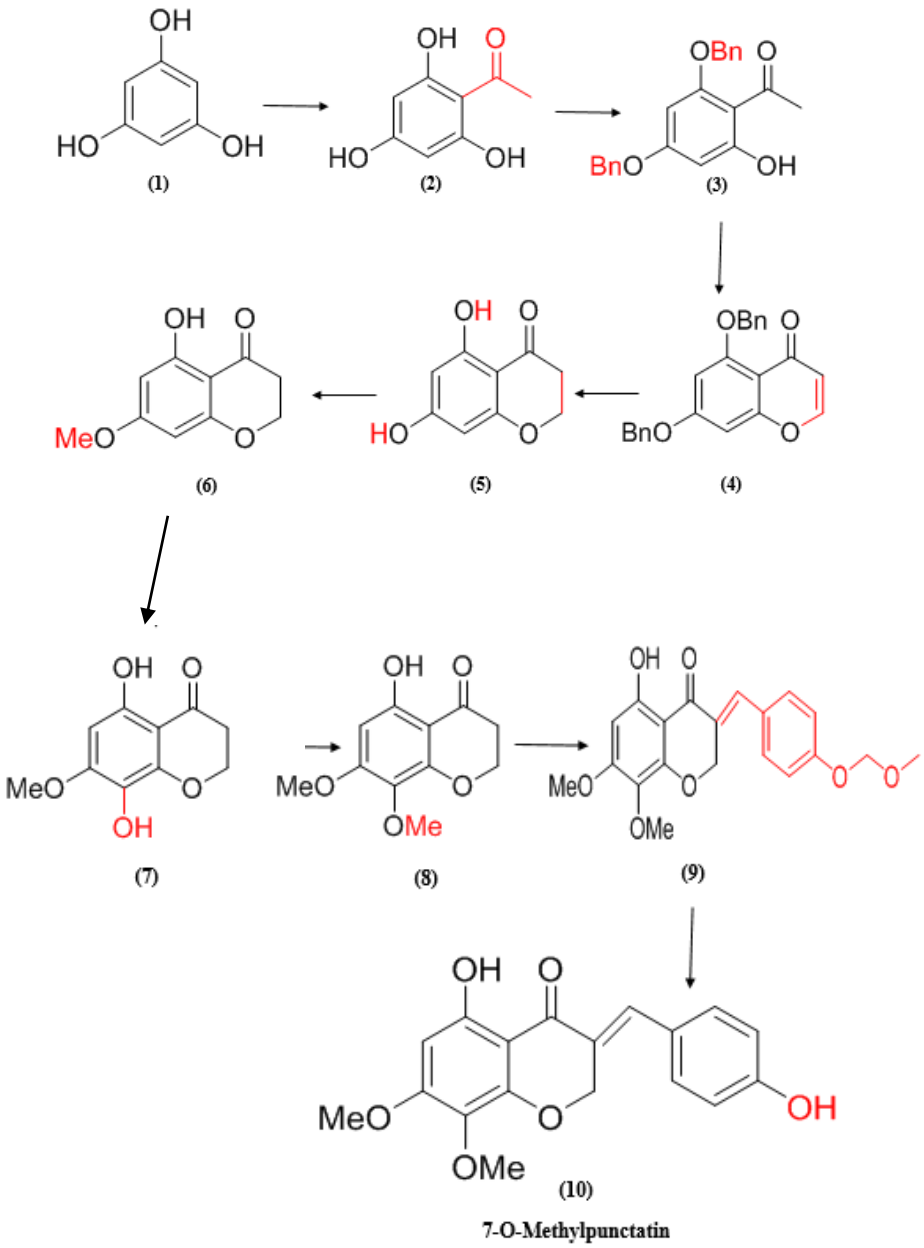

Figure 7 Synthesis route of 7MP

1. *The 1H- NMR spectrum of synthesized 7MP*

The ^{1}H NMR spectrum of 7MP exhibits the following types of signals; a) two sharp singlets around 9.7 ppm and 8.3 ppm corresponding to the secondary amine groups protons for the hydrazide; b) a broad singlet between 6.3-6.5 ppm corresponding to the primary amine groups

protons for the triazine; (c) two characteristic triplets around 2.6 ppm and 4 ppm, corresponding to the two methylene group protons.

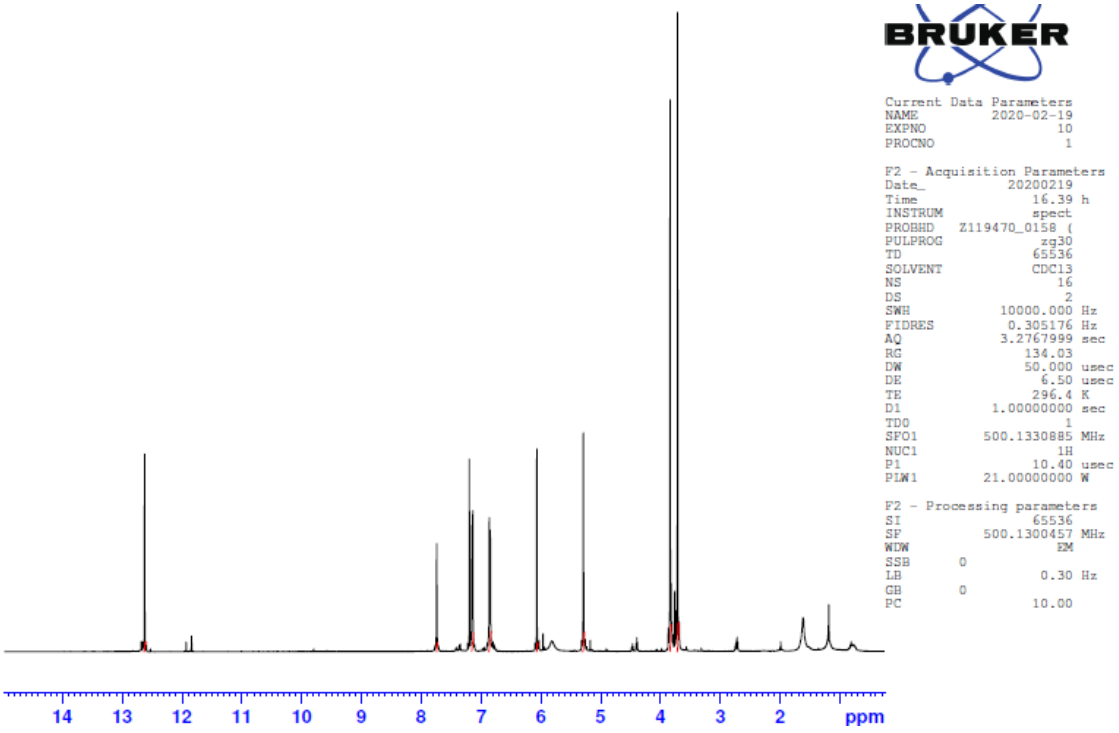

Figure 8 H^1NMR Spectrum for 7MP

2. X-ray Crystallography of 7-O-Methylpunctatin

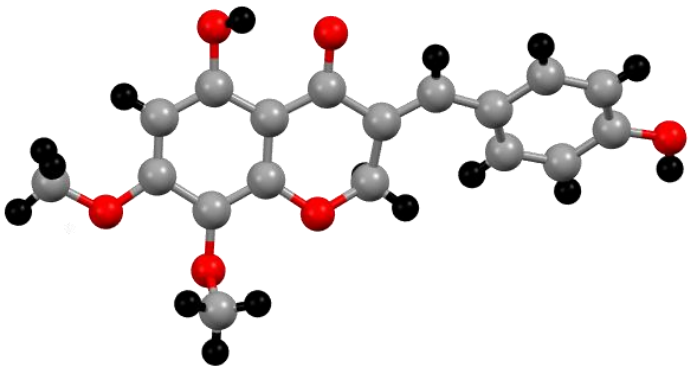

Figure 9 Crystallography structure of 7MP

3. Effect of 7MP on the viability of MCF-7 and MDA-MB-231 cells.

Cell viability by MTT assay was evaluated in MCF-7 and MDA-MB-231 breast cancer cell lines treated with 7MP at various concentrations (0, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM). 7MP decreased the viability of both the MCF-7 and MDA-MB-231 cell lines in a concentration and time-dependent manner. Indeed, with the lowest concentration (20 µM), 7MP attenuated cellular viability to 60 ± 2.80 % and 71.79 ± 2.80 % in MDA-MB-231 and MCF-7 cells respectively after 72 hours (**Figures 8-9**). This suggests that 7MP exerts an anti-proliferative effect on breast cancer cells.

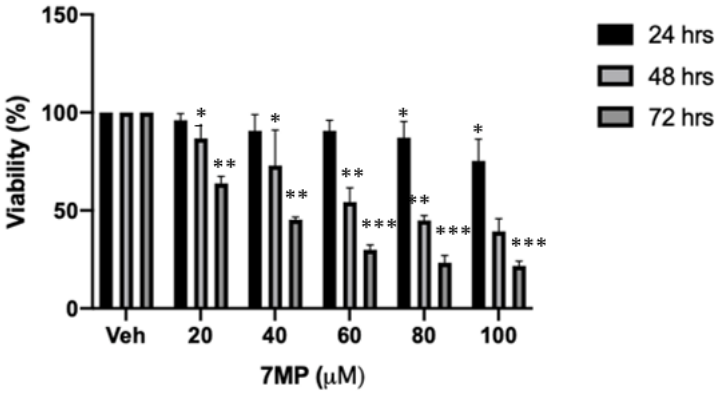

Figure 10 7MP decreases the viability of MDA-MB-231 cells in a time and concentration-dependent manner

MDA-MB-231 cells were treated with increasing concentrations of 7MP (20, 40, 60, 80 and 100 μM) for 24, 48, 72 hours, and cellular viability was determined by MTT. *p < 0.05, **p < 0.01, ***p < 0.005. Two-way ANOVA was performed.

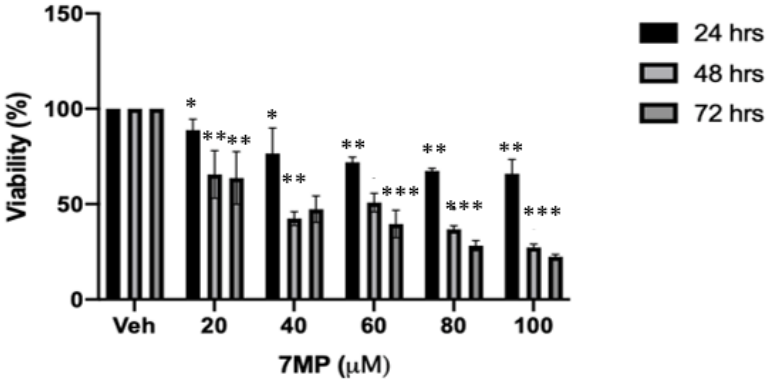

Figure 11 7MP decreases the viability of MCF7 cells in a time and concentration-dependent manner.

MCF-7 cells were treated with increasing concentrations of 7MP (20, 40, 60, 80 and 100 μM) for 24, 48, 72 hours, and cellular viability was determined by MTT. *p < 0.05, **p < 0.01, ***p < 0.005. Two-way ANOVA was performed.

4. 7MP affects cell cycle progression

After determining the anti-proliferative effect of 7MP on breast cancer cells, we then sought to examine the effect it has on the cell cycle progression. As seen in the (**Figure 10**), there is a G0/G1 arrest in MDA-MB-231 cells treated with 7MP (20 µM). This means that 7MP reduces the growth of breast cancer cells by G0/G1 cell cycle arrest.

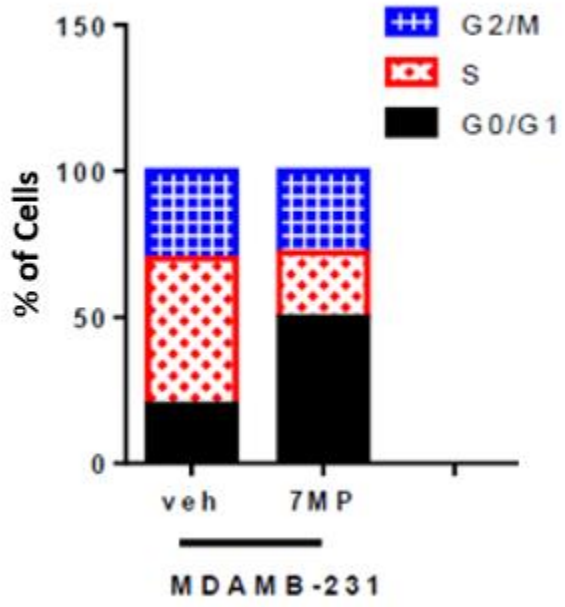

Figure 12 7MP induces cell cycle arrest at G0/G1.

MDA-MB-231 cells were treated with 20 µM of 7MP for 48 hours. Then, cells were stained with PI and flow cytometry was performed. Data represent the mean of three independent experiments.

5. 7MP targets COX-2 but not COX-1 expression.

It is important to investigate whether 7MP decreases the protein levels of both COX-1 and COX-2. Therefore, MDA-MB-231 cells were treated with or without 7MP (20 µM) for 24 hours and protein levels were determined by western blot. Interestingly, results showed that

7MP significantly decreased the protein levels of COX-2 (from 1 to 0.42 folds) but not COX-1 (1 to 1.07 folds) in MDA-MB-231 cells (**Figure 11**). This means that 7MP targets specifically one of the two isoforms of cyclooxygenase enzyme; namely COX-2.

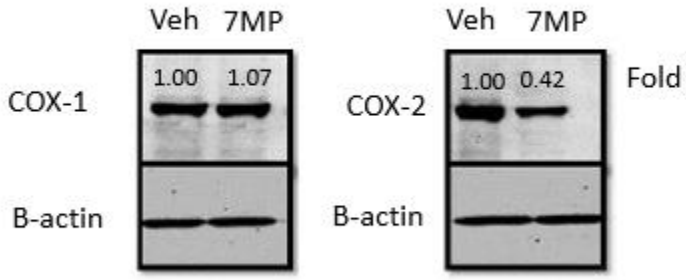

Figure 13 7MP decreases COX-2 but not COX-1

MDA-MB-231 cells were treated with 20 μM of 7MP for 24 hours. Then, expression levels of COX-1 and COX-2 were determined by using western blotting with levels of β actin as control. Values represent mean of fold change of three independent experiments.

6. 7MP decreases the amount of released PgE2 in MDA-MB-231.

PgE_2 is the major tumorigenic product of COX-2 in breast cancer. So, we sought to determine whether 7MP could affect the levels of PgE2. Interestingly, there is a significant decrease of PgE_2 from 7MP treated cells compared to the vehicle treated ones in both the parent and COX-2 overexpressing cells (**Figure 12**). This further validates that 7MP acts against COX-2 or at least a COX-2 mediated pathway.

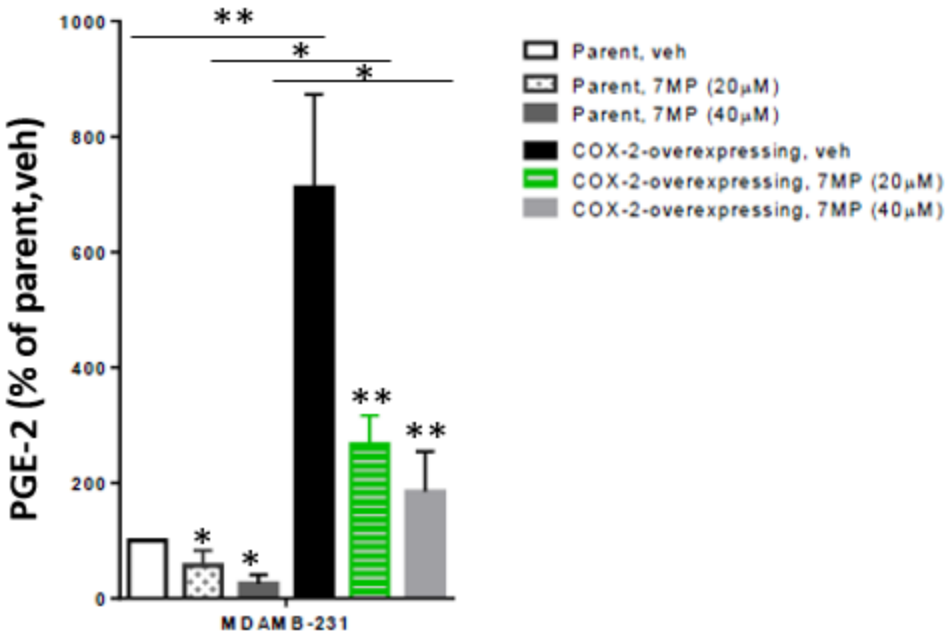

Figure 14 7MP decreases the amount of PgE2 released in MDA-MB-231

MDA-MB-231 (parent and COX-2 overexpressing) cells were treated with 20 and 40 μM of 7MP for 24 hours. Then, the secreted levels of PgE2 were determined by using ELISA. Data represent the mean of three independent experiments ± SEM. One-way ANOVA was performed, *$p < 0.05$, and **$p < 0.01$.

7. 7MP affects apoptotic markers.

When DNA repair fails, apoptosis which is also known as programmed cell death occurs. Caspases are the family of proteases that play an intricate role in apoptosis. Caspases 3, 6, and 7 are three "executioner" caspases that carry out the major proteolysis activity that lead to apoptosis. Since we showed that 7MP caused a cell cycle arrest in MDA-MB-231 cells we then wanted to check for the level of caspases. We also wanted to examine the effect of COX-2 overexpression on the level of caspases.

As seen in the (**Figure 13**), overexpressing COX-2 decreased the activities caspases 3/7 compared to the parent cell line. However, 7MP significantly increased the actvities of

caspases 3 and 7 in both parent and COX-2 overexpressing cell lines in a concentration-dependent manner. Thus, 7MP induces apoptotic cell death in MDA-MB-231 cells and in the COX-2 overexpressing ones.

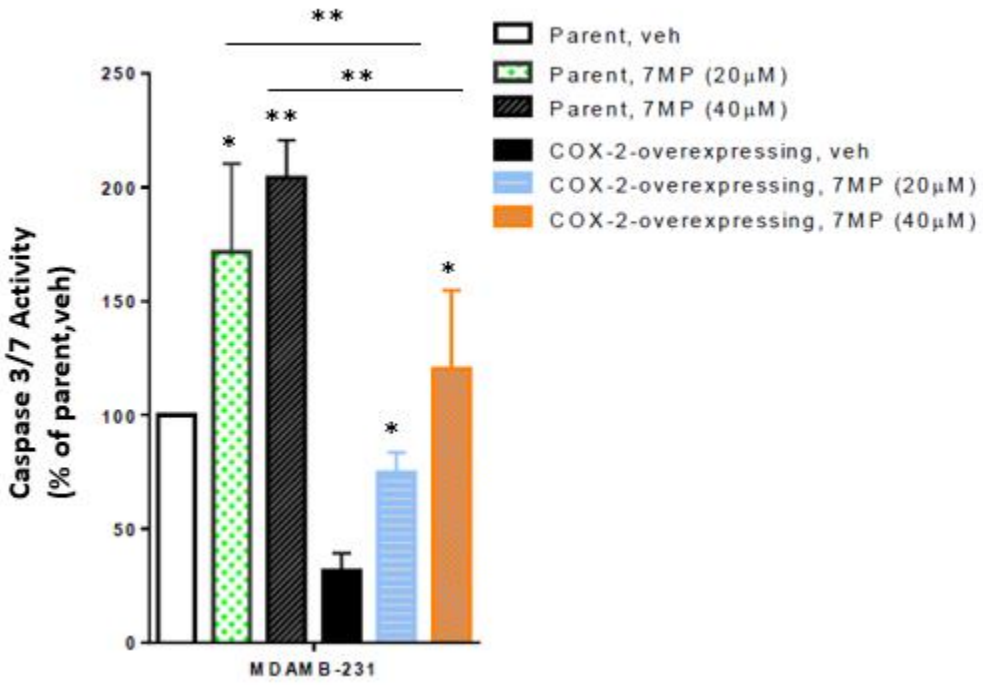

Figure 15 7MP induces caspase3/7

MDA-MB-231 (parent and COX-2 overexpressing) cells were treated with 20 and 40 μM of 7MP for 48 hours. Then, levels of caspase3/7 were determined by using caspase-glo assay. Data represent the mean of three independent experiments as mean ± SEM. One-way ANOVA was performed, *$p < 0.05$, and **$p < 0.01$.

8. 7MP inhibits migration of MCF-7 cells.

Next, we examined the effect of 7MP on the migratory potential of cells by using the wound healing scratch assay. In fact, treatment of cells with 20 μM significantly attenuated cellular migration within 2 hours as compared to control (**Figure 14**). In addition, this anti-migratory effect was in a time-dependent manner. Thus, 7MP could induce anti-migratory effect on MDA-MB-231 cells.

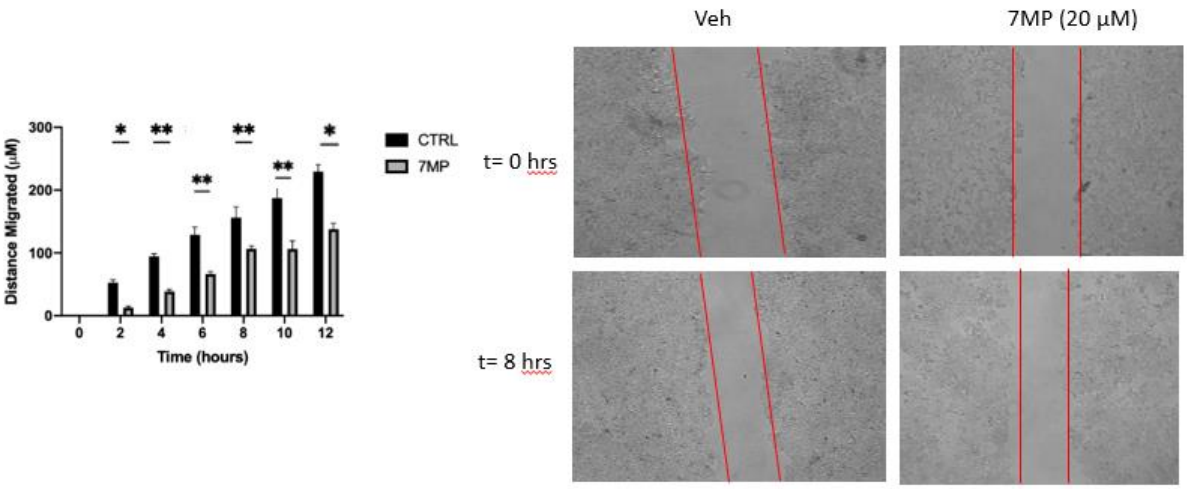

Figure 16 7MP inhibits the migration of MCF7 cells in a time dependent manner.

MCF7 cells were treated with 7MP (20 μM) and cell migration was assessed using wound healing assay. Distance migrated of MCF7 cells were represented as mean ± SEM. *p < 0.05 and **p < 0.01. One-way ANOVA was performed. Data represent the mean of how many? independent experiments ± SEM.

9. 7MP inhibits basal and COX-2 induced NF-κB activity in MDA-MB-231 cells.

Nuclear factor-κB (NF-κB) is a transcription factor that is considered one of the major molecules that link inflammation and cancer. So, we wanted to evaluate the effect of 7MP on the activity of the pro-inflammatory molecule NF-κB. COX-2 overexpression increased NF-

κB activity significantly which is expected and aligns with the literature (**Figure 15**).Importantly, 7MP was able to inhibit both the basal and COX-2 induced NF-κB activity in MDA-MB-231 cells.

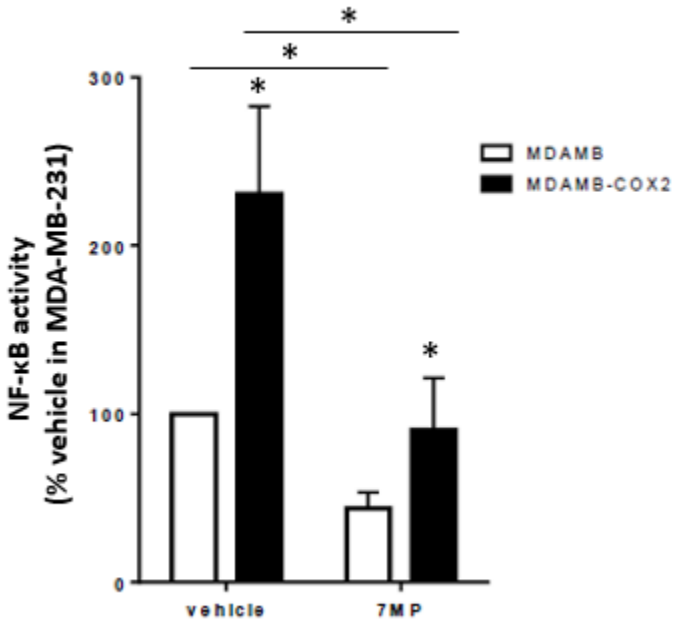

Figure 17 7MP inhibits basal and COX-2 induced NF-κB activity in MDA-MB-231 cells

MDA-MB-231 (parent and COX-2 overexpressing) cells were treated with 20 μM of 7MP. Then, NF-κB activity was determined by using the luciferase assay. Here the vehicle treated is MDA-MB-231 parent cell line are considered 100 to which every other group is compared to. Data represent the mean of three independent experiments ± SEM. One-way ANOVA was performed, *$p < 0.05$.

10. 7MP inhibits basal and COX-2 induced ERK1/2 in MDA-MB-231 cells.

The increased activity of the MAPK group ERK1/2 is associated with a malignant phenotype. ERK1/2 gets activated upon different signals including inflammation which is further pronounced in MDA-MB-231 cells over expressing COX-2. Indeed, COX-2 overexpression increased the level of phosphorylated ERK to total ERK. In addition, 20 μM treatment of 7MP did attenuate both basal and COX-2 induced activation of ERK. Therefore, 7MP could exert its anti-cancer effect through inhibiting the ERK1/2 pathway.

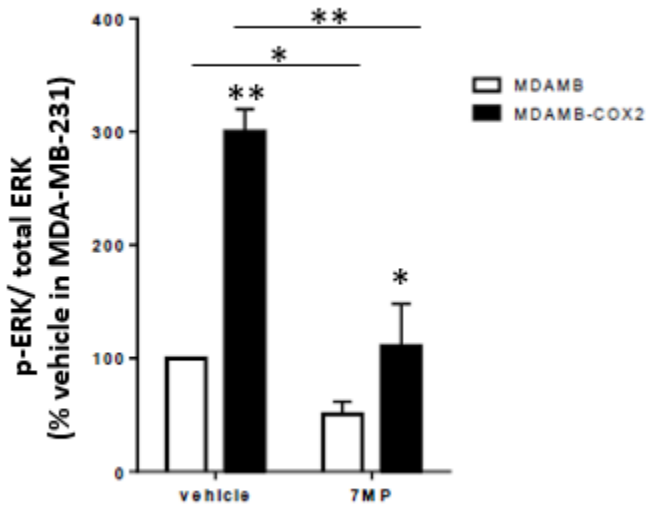

Figure 18 7MP inhibits basal and COX-2 induced ERK1/2 in MDA-MB-231 cells

MDA-MB-231 (parent and COX-2 overexpressing) cells were treated with 20 μM of 7MP. Then, pERK/total ERK activity was determined by using the In-cell ELISA. Here the vehicle treated is MDA-MB-231 parent cell line are considered 100 to which every other group is compared to. Data represent the mean of three independent experiments. One-way ANOVA was performed, *p < 0.05, and **p < 0.01.

11. 7MP inhibits angiogenesis in CAM model.

Angiogenesis is a crucial process in tumor development and metastasis. Thus, we wanted to check the effect of 7MP on the formation of blood vessels by using the CAM model. Interestingly, as shown in (**Figure 17**), 7MP significantly inhibited angiogenesis in this model which is depicted by the strikingly significant drop in vessel area (-20.68 ± 3.75 % for 7MP-treated vs 8.85 ± 3.49 % for control) and number of junctions (-32.68 ± 13.75 % for 7MP-treated vs 8.85 ± 3.49 % for control) after 24 hours of treatment. Thus, these results indicate that 7MP inhibits angiogenesis in-ovo.

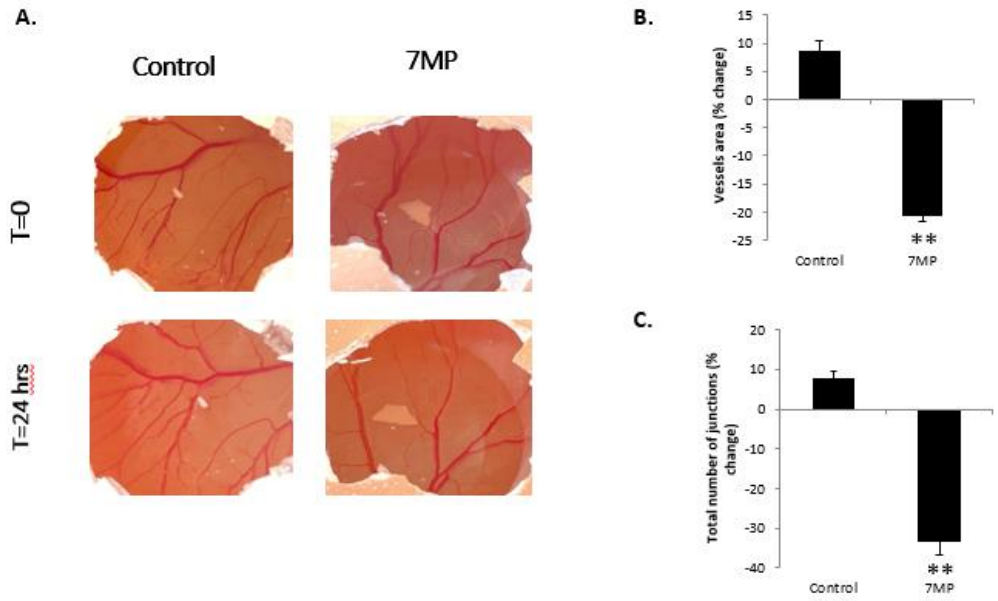

Figure 19 7MP inhibits angiogenesis in-ovo

7MP (20 μM) was added on the CAM of EB6 embryo. Photographs (A) taken 24 h after treatment show the change in vessels area (B) and the total number of junctions (C). Values are represented as mean ± SEM. **p < 0.01.

CHAPTER IV

DISCUSSION

Breast cancer remains the most common type of cancer in females worldwide [150]. In developed countries, diet-related factors are held accountable for around 30% of all cancers including breast cancer. The consumption of fruits, vegetables, legumes and green tea have appeared to be protective against cancer; a property attributed to the phytochemicals found in these plants [151, 152]. Moreover, epidemiological and pre-clinical evidence suggest that polyphenolic phytochemicals have chemo-preventative properties against breast cancer [151]. In fact, phytochemical polyphenols have been reported to act as antioxidants, decrease the proliferation of cancer cells and block pro-inflammatory cytokines and transcription factors that mediate cancer progression [153]. All in all, these properties culminate in these compounds' ability to reduce inflammation and cancer recurrence [153].

Homoisoflavonoids are polyphenolic phytochemicals that possess great potential yet are still poorly investigated in the context of breast cancer. Here, we report the synthesis of a homoisoflavonoid previously isolated from the plant *bellevalia eigii* and named "7-O-methylpunctatin". We then evaluated its biological activity against breast cancer cell lines in regular and 'inflamed-like' conditions.

Clinical, epidemiological and experimental studies all support the notion that inflammation and cancer are tightly related [154, 155]. Among the major mediators of cancer and inflammation are NF-κB, TNF, and COX-2. These molecular players promote inflammation and tumor progression through the enhancement of cancer cell proliferation, metastasis and anti-apoptotic activity [156]. Of these players, COX-2 is over expressed in different cancers including breast cancer. COX-2, unlike COX-1, is detected in tumor breast

tissue but not in normal tissue [157]. It is overexpressed by around 40% in tumor tissue and by around 80% in ductal carcinoma in situ (DCIS) [158]. Celecoxib, a selective COX-2 inhibitor, has been associated with the reduced risk and prevention of breast cancer [159]. However, a major drawback of celecoxib use is its associated cardiotoxicity [160]. Results from our laboratory demonstrated that 7MP prevents both platelet aggregation (data not published) and dedifferentiation of micro VSMCs implying a cardioprotective advantage [148]. Interestingly, in this report we show that 7MP specifically targets COX-2 but not COX-1 in the aggressive breast cancer cell line MDA-MB-231.

COX-2 converts arachidonic acid into PGE_2 [161]. PGE_2 in itself has been shown to promote breast cancer through: immunosuppression [162], angiogenesis [163], stem-like cell (SLC) formation [164], and migration [165]. Here, we report that 7MP reduces PGE_2 release from breast cancer cells.

7MP exhibited an anti-proliferative effect against the two breast cancer cell lines: MDA-MB-231 and MCF-7. The cytotoxic effect of 7MP on MCF-7 cells (ERα (+)) and MDA-MB-231 cells (ERα (-)) was observed in a time and concentration-dependent manner. Notably, a major problem faced when examining the anti-breast cancer properties of phytochemicals is that they might exert a biphasic effect on estrogen-dependent cancer models by activating ERα receptors[166, 167] . This major drawback is not observed in our study since no proliferative effect was observed by 7MP on both cell lines at a wide range of concentrations of 7MP.

The tumor microenvironment employs pro-inflammatory molecules such as ROS, cytokines, inducible nitric oxide synthase (iNOS), and NF-κB to provide a suitable niche for cancer cell growth [168]. Different nuclear transcription factors including NF-κB can

upregulate the constitutive expression of COX-2 due to the various binding sites at the promoter region of the *COX-2* gene [169]. Here we show that 7MP inhibits basal and COX-2 induced NF-κB activity. Importantly, COX-2 is known to activate one of the major components of the tumor microenvironment; the matrix metalloproteinases (MMPs) in an NF-κB dependent manner [170]. MMPs degrade the components of the extracellular matrix (ECM) and therefore are needed for the migration and invasion of cancer cells. In addition, a prerequisite step for cancer cell migration is the adhesion to components of the ECM. The expression of the receptors for the adhesion molecule "integrin" on the surface of breast cancer cells has been associated with different metastatic behavior [171]. Previous studies have shown that an interaction between the ECM proteins "fibronectin" and "vitronectin" with integrins causes an elevation in the expression of more than 30 genes involved in tumor progression [172, 173]. Therefore, the inhibition of the adhesion of integrins to fibronectin has been shown to decrease invasion and migration of breast cancer cells [174]. 7MP decreased the migratory capacity of MCF-7 and MDA-MB-231 cells as seen in the wound healing experiment. It is expected that the levels of MMPs and adhesion of breast cancer cells to the components of the ECM would decrease upon the treatment with 7MP however this still needs to be confirmed the proper assays.

The irregularity in the MAPK signaling pathways, allows for the initiation and progression of various malignancies. Among the three major MAPK groups, ERK1/2 is the most relevant to breast cancer. It can be activated by different factors including hormones, oxidative stress, and inflammatory cytokines [175]. Upstream of ERK1/2, the interplay between hormones and the PI3-K-dependent pathways activate Akt and PKC zeta [176]. This activation then leads Akt to increase the transcription of cyclin D1 and PKC zeta, allowing the

activation and nuclear translocation of ERK2 [177]. This interplay of signaling pathways with cell cycle regulators allows for the cells to enter the S phase leading to increased cellular proliferation and tumor growth [176, 177]. In 50% of breast tumors, MAPK signaling is more active compared to the benign tissues surrounding them [175]. Numerous investigators have shown that, the transfection of breast cancer cells with COX-2 elevated ERK1/2 phosphorylation level compared to the non-transfected cells. Excitingly, 7MP was able to reduce the phosphorylation of ERK1/2 in basal and COX-2 induced conditions of breast cancer cells.

In addition, the ERK/MAPK pathway is involved in cell cycle progression [178]. The multiple checkpoints during the cell cycle are present to ensure that damaged DNA is not transmitted to daughter cells; however, if DNA is not repaired, apoptotic cascade is activated [179]. Both ERK1 and ERK2 enhance the transcription of cyclin D1 by inhibiting its transcription corepressor TOB, which is a critical protein for the progression through the G1 phase [180]. In fact, cyclin D1 has been reported as an oncogene that is elevated by ~50% in human breast cancers allowing these cells to evade the G1 checkpoint [181]. The treatment of MDA-MB-231 cells with 7MP caused a G0/G1 arrest and we speculate that it is through decreasing ERK1/2 activity and consequently affecting cyclin D1 transcription. Further examination of p27 and p53 proteins is warranted in order to investigate the players our compound utilizes in causing this cell cycle arrest.

Programmed cell death or apoptosis is an essential process that rids the body of abnormal cells in order to keep the organism stable. However, in the case of carcinogenesis cancer cells escape it and further promote the malignant phenotype. Apoptosis generally occurs when the genome repair initiated by the tumor suppressor protein p53 fails, in order to

prevent the transmission of damaged DNA [182]. The genes involved in apoptosis include the antiapoptotic bcl-2 and the pro-apoptotic bax [183]. Apoptosis occurs when bcl-2/bax ratio is low [184].The mechanism in which bcl-2/bax ratio leads to apoptosis is related to cytochrome c, a molecule that activates the caspase cascade when released from the mitochondrial membrane [185]. Moreover, caspases are critical mediators of apoptosis through mitochondrial events [185]. We therefore investigated the effect of 7MP treatment on caspases 3 and 7. Evidently, 7MP increases the levels of caspases 3 and 7 which are the early markers of apoptosis.

Finally, a process that primes tumors for proliferation, progression and metastasis is angiogenesis [186]. This process of vessels sprouting from pre-existing ones is controlled by angiogenesis inducers (such as growth factors, adhesion molecules, chemokines) and angiogenesis inhibitors (such as endostatin and canstatin) [187]. VEGF is the most prominent family in the process of angiogenesis with VEGF-A being the most investigated member [188]. Bevacizumab, an antibody that targets VEGF-A, is an FDA-approved anti-angiogenic drug [189]. For this purpose, we used the chick chorioallantoic membrane (CAM) model to assess for anti-angiogenic activity of 7MP. Interestingly, we found that there is a significant inhibition in blood vessel sprouting when the CAM model is treated with 20µM of 7MP. This is promising because although there are several drugs that aim to inhibit angiogenesis via targeting VEGF such as bevacizumab (Avastin), they are associated with side effects and resistance [139]. Finding a molecule that could target VEGF and hence be used with existing anti-VEGF therapy or as anti-VEGF therapy on its own is therefore favorable in the treatment of breast cancer.

CHAPTER V

CONCLUSION

The overexpression of COX-2 in breast cancer cells potentiated the malignant phenotype by increasing proliferation, ERK1/2 activation, NF-κB activity, and decreasing the levels of apoptotic caspases. Excitingly, our study suggests that 7MP could reverse this exaggerated malignant phenotype caused by COX-2 overexpression. Moreover, 7MP was also able to attenuate the basal migratory, adhesive, and proliferative capacities of breast cancer cells. 7MP also caused breast cancer cells to accumulate in G0/G1 and increased the levels of apoptotic markers. Lastly, 20 μM of 7MP were able to attenuate angiogenesis in CAM model. What is reported here is an indicative of the great potential 7MP possesses in attenuating the basal and COX-2 induced malignant phenotype of breast cancer cells.

CHAPTER VI

LIMITATIONS AND FUTURE PERSPECTIVE

- Loss of function experiments should be done to check for the role of EP4 receptors and if it indeed acts through COX-2/PGE2/EP4 pathway.
- Use 7MP treatment along with other breast cancer chemotherapeutic drugs in order to check if they act synergistically or not.
- We did not use multiple cell lines for all experiments; we therefore opt to test 7MP on a battery of breast cancer cells with different receptor expressions.
- We did not test the pharmacokinetic profile of this compound and whether it could be used in more advanced models.
- We need to perform binding assays to check for the specific target of our compound.
- We should test the compound on a wider array of molecules than what we already did (check its effect on interleukins, NO production, MMPs, EMT markers etc.)
- Compare 7MP effect with Celecoxibs and NSAIDs.
- If the compound did show promising effects on a wide battery of breast cancer cell lines, in-vivo experiments should be opted for next.

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