CHARACTERIZATION OF THE EFFECTS OF 3,3'-DIINDOLYLMETHANE (DIM) IN THE TRAMP MOUSE PROSTATE CANCER MODEL AND DIM'S INTERACTION WITH ESTROGEN RECEPTOR SIGNALING

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CHARACTERIZATION OF THE EFFECTS OF 3,3'-DIINDOLYLMETHANE (DIM) IN THE TRAMP MOUSE PROSTATE CANCER MODEL AND DIM'S INTERACTION WITH ESTROGEN RECEPTOR SIGNALING

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ABSTRACT

3,3'- diindolylmethane (DIM) is an acid -derived compound formed during the digestion of indole-3-carbinol (I3C), which is one of the main compounds in cruciferous vegetables, such as broccoli, cabbage and kale. Studies indicate that the cancer protective effects of cruciferous vegetables and I3C partially rely on DIM, which is the bioactive form of I3C in the body, and DIM has been shown to interact with several signaling pathways in cancer. However, the primary molecular working mechanism of DIM is still not clear. In this dissertation, we hypothesize that DIM works through estrogen receptor (ER) signaling to inhibit prostate cancer. We focus on DIM's interaction with ER, as well as its

effects on preventing advanced prostate cancer and weight gain in the TRansgenic Adenocarcinoma of Mouse Prostate (TRAMP) model.

Here we show that DIM stimulates ER activity. Though DIM was reported to be an ERβ selective agonist in the literature, we found that DIM stimulated both ERs' transcriptional activities in luciferase reporter assays performed in different cell lines. To assess for the role of ER in DIM's function of inhibiting cancer cell growth, DIM was tested in different cell lines with various ER expression pattern combinations. DIM did not inhibit growth of an ERα-only cell line at the concentrations at which it would normally inhibit in cell lines expressing ERβ, suggesting ERβ was involved in the growth inhibition by DIM.

DIM dose-dependently inhibited the growth of TRAMP-C2 cells, a cell line derived from a prostate tumor of a TRAMP mouse. In order to evaluate the ability of DIM to inhibit advanced prostate cancer in the *in vivo* TRAMP mouse model, at the relative concentrations used *in vitro*, we fed a high fat diet to TRAMP mice with/without the addition of 0.04%, 0.2% and 1% DIM to the diet. Compared to the high fat diet alone, both 0.2% and 1% DIM dose significantly decreased the incidence of poorly differentiated carcinoma (PDC) in our TRAMP mice (60% vs.38% and 24%, respectively). A nice trend line of decreasing PDC incidence while increasing DIM dose

was observed, which paralleled what we saw *in vitro*.

A few papers reported the effect of DIM on weight gain. In our study, DIM had an effect on mice body weights. Mice fed with 1% DIM had an average body weight significantly lower than other groups, primarily because of an initial weight loss when the diet began. Statistical analysis excluded the potential effects of weight loss on PDC incidence, showing that PDC incidence was only negatively correlated with DIM dose ($p=0.004$), but not body weight ($p=0.998$). Since ERβ was reported to alleviate obesity, we still wanted to know whether the weight loss was caused by DIM's activation of ERβ. Mice fed with 1% DIM diet presented with a dramatic weight drop in the first week on study, then recovered, but were never able to catch up with mice in the other groups. Because of reports in the literature that ERαKO and ERβKO mice genotypes will affect long term body weight, we were curious if DIM might be acting to influence weight loss through an estrogen receptor. Thus, we designed experiments either by feeding or intraperitoneally injecting DIM into ER knockout mice to examine the initial weight drop in mice. We found that the initial weight drop was irrelevant to mice ER genotype nor DIM injection, but only related to being orally fed DIM. Together with the lower food intake in mice fed with 1% DIM compared to the control, we concluded that the initial weight loss was caused by mice disliking the taste of DIM, which smells like "mothballs".

Overall, our study showed that DIM could prevent advanced prostate cancer in a dose dependent manner and the prevention was potentially, at least in part, through modifying ERβ activity. Future DIM studies are needed *in vivo* using double transgenic ERKO/TRAMP mice to confirm this hypothesis that ERβ plays a key role in DIM's prostate cancer prevention ability.

CHAPTER I

LITERATURE REVIEW

This dissertation focuses on dietary components, including botanical compound 3’3-diindolylmethane (DIM) and cholesterol, their effects on prostate cancer prevention and treatment, as well as their interactions with the Estrogen receptor (ER) signaling pathway. In the following review, a brief introduction of prostate cancer and its detection and therapy will be discussed, followed by a description of the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) prostate cancer model. Estrogen receptor isoforms and their opposing functions in preventing cancers will be described, as they relate to a broccoli compound DIM and its relationship with cancer.

Prostate Cancer

Prostate cancer is the most prevalent cancer among U.S. males. About 11.6% of men will be diagnosed with prostate cancer during their lifetime[1]. In 2017, it was estimated that there were 161,360 new cases of prostate cancer, which accounts for almost 1 out of 5 new diagnoses, and about 26,730 people will die from this disease, making it the third leading cause of cancer death in men [2]. Prostate cancer incidence increases with age. 99.5% of prostate cancers are diagnosed in men over 45 years old and the median age at

diagnosis is 66 years.

Prostate cancer was first discovered in 1853. A surgeon named J. Adams at The London Hospital found this during a histological examination and noted it as “a very rare disease”[3]. However, it was not until the early 20th century that prostate cancer was differentiated from other types of urinary obstruction[4]. Since then, the incidence of prostate cancer has dramatically increased, which also lead to remarkable changes in screening and treatment of prostate cancer. This increased incidence may be due to an ascertainment bias since it was not recognized before. Additionally, prolonged life expectancy may also contribute to the increased prostate cancer incidence because prostate cancer increases rapidly with age.

Prostate Cancer Screening

Nowadays, routine screening for prostate cancer includes digital rectal examination (DRE) and prostate-specific antigen (PSA) blooding testing. DRE was first used in the 1970s and use of PSA as a detection tool for prostate cancer was approved by the United States Food and Drug Administration (FDA) in 1994[5]. Both of them have contributed to early detection of prostate cancer. However, there are some limitations or even potential harms making it controversial. One of the major concerns is that PSA test could cause over-diagnosis and over-treatment. Some tumors detected by PSA test grow very slowly

and will not threaten a man's life. Detecting those tumors can expose men to over-treatment, causing unnecessary side-effects from the treatments and also creating anxiety for the patients. Another concern is that the PSA test has a high possibility of giving a false-positive result. This leads to additional medical procedures, such as prostate biopsy, together with side-effects from the procedure. Only 25% of men, who have elevated PSA level, are found to have prostate cancer after the prostate biopsy[6]. Several randomized controlled trials of prostate cancer screening have been conducted in recent years[7-10]. However, there is inadequate evidence showing if screening for prostate cancer through the PSA test or DRE can reduce mortality from prostate cancer. Researchers are investigating new ways of screening prostate cancer, which can distinguish slow-growing tumors from fast-growing, lethal cancers. Some new tests based on other forms of PSA or biomarkers includes: the phi (combines results of total PSA, free PSA and proPSA, which is the precursor of PSA), the 4Kscore test (combines results of total PSA, free PSA, intact PSA and human kallikrein 2), urine prostate cancer antigen 3 (PCA3), urine TMPRSS2:ERG gene fusion and ConfirmMDx, which checks a panel of genes in a prostate biopsy sample thus helping rule in patients with false negative biopsy results to further repeat biopsies or treatment as well as rule out cancer-free men from undergoing repeat biopsies.

Prostate Cancer Treatment

There are three primary options for men diagnosed with localized prostate cancer: expectant management (consists of watchful waiting and active surveillance), surgery and radiation. For men with metastatic prostate cancer, androgen deprivation therapy (ADT) is the first-line treatment. The goal is to block the androgen dependent grow of prostate cancer. There are several types of ADT.

First, we will discuss the luteinizing hormone releasing hormone (LHRH) agonists and antagonists. LHRH induces production of LH by the pituitary. LH binds to the LH receptor in the testis and then induces production of testosterone. LHRH antagonists, such as degarelix, bind to LHRH receptor directly and then inhibit the concentration of LH and testosterone; while LHRH agonists, such as leuprolide, goserelin, triptorelin and histrelin downregulate the LHRH receptor by feedback inhibition, thus decreasing LH and testosterone production.

Though LHRH agonists and antagonist can inhibit androgen synthesis by the testis, other cells in the body, for example, adrenal gland or even prostate itself [11], can still make androgen and fuel the growth of prostate cancer. In this case, some other treatments are introduced to inhibit androgen synthesis. One of them is 5α Reductase inhibitor, which blocks the conversion of testosterone to DHT [12-15]. Another example is CYP17

inhibitor, such as abiraterone acetate, working through inhibiting production of the testosterone precursors, dehydroepiandrosterone (DHEA) and androstenedione, thus decreasing testosterone and DHT concentrations [16-18].

Another type of ADT works not by lowering androgen levels, but by preventing androgens from working. In this case, drugs are called anti-androgens or antagonist. Anti-androgens, such as flutamide, bicalutamide and nilutamide bind to androgen receptor (AR) and prevent androgen's binding. Enzalutimide (MDV3100), discovered by Charles Sawyers and approved in 2012 by the FDA, is a newer type of anti-androgen. It inhibits androgen receptor signaling via three stages: competitive binding inhibition of androgens to AR, preventing the translocation of AR to nucleus, and impairing both the binding of AR to DNA and recruitment of coactivators by inducing a conformational change of AR [19-22].

In many cases, prostate cancer develops and becomes unresponsive to ADT, leaving prostate cancer patients with fewer treatment options. Chemotherapy drugs such as docetaxel, which works by interfering with the normal function of microtubules and thus preventing cell division, are used to treat advanced prostate cancer and have been shown to improve survival [23, 24]. Sipuleucel-T (Provenge), is the first FDA approved prostate cancer vaccine in the United States. This vaccine is made specifically for each man, with

white blood cells harvested from patient and then incubated with fusion protein antigen, consisting of prostatic acid phosphatase (PAP) and granulocyte-macrophage colony stimulating factor (GM-CSF). Those activated cells are then reintroduced to the patient, which helps the immune system attack the prostate tumor and results in median survival increaseing 4.1 months compared to placebo [25-28]. Bone is the major site of prostate cancer metastasis. When prostate cancer metastasizes to the bone, osteoclasts often becomes over-activated, which then dissolves the bone, making holes in the bone called lytic lesions and causing bone to break more easily. Additional treatments, for example bisphosphonates and denosumab, are used to treat prostate cancer metastasis in the bone by inhibiting osteoclasts and have been shown to improve bone health and bone metastases survival[29, 30].

TRAMP Mouse Prostate Cancer Model

The **Tr**ansgenic **A**denocarcinoma of the **M**ouse **P**rostate (TRAMP) model was first introduced by Greenberg and colleagues in 1996. TRAMP was developed by putting SV40 large and small T-antigen oncogene under the control of the rat probasin (rPB) gene promoter [31]. rPB is specifically expressed in prostate epithelium and it was shown that the -426 to +28 region of rPB promoter was the key element to drive androgen-responsive expression of the probasin gene[32]. In the TRAMP model, the PB-SV40 T-antigen (PB-Tag) is expressed in the dorsal and ventral prostate lobes. At the

onset of puberty, SV-40 T-antigen binds and inactivates the p53 and retinoblastoma (Rb) tumor suppressors. Prostate then undergoes unregulated growth and mice spontaneously develop prostate cancer, that metastasizes to many of the same organs found in human prostate cancer metastasis.

There are six stages of histological grading of prostate tumors in the TRAMP model: 1) normal, 2) hyperplasia, 3) prostate intraepithelial neoplasia (PIN), 4) well-differentiated carcinoma (WDC), 5) moderately-differentiated carcinoma (MDC), and 6) poorly-differentiated carcinoma (PDC) (Figure I-1). Stages 1-3 are considered to be non-cancerous. Stages 4 and 5 are cancers that are less aggressive, while stage 6 is the most aggressive cancer. Based on the findings of our collaborator Dr. Cynthia Besch-Williford, we have noted that PDC can arise independently as small foci and with enough time, it can overwhelm the whole prostate gland. When scoring the prostate, if even a small portion of the prostate shows a poorly differentiated morphology, we score it as a PDC. What's more, a neuroendocrine biomarker synaptophysin is only found to be expressed in PDC but not in WDC, indicating they may arise from different cell lineages. Neuroendocrine prostate cancer is usually considered to be AR negative. However, at least 50% of the synaptophysin-positive PDC expresses AR. CRPC patients after prolonged use of AR antagonists were found to express neuroendocrine biomarkers with reduced AR expression, suggesting there is a potential trans-differentiation from

AR-positive carcinoma to AR-negative neuroendocrine prostate cancer [33]. In addition, tumorigenesis of PDC in TRAMP was shown to be AR independent, with 8 out of 10 mice which were castrated at 12 weeks, developing PDC at 24 weeks [34]. Taken together, these properties make TRAMP a good model to study castration resistant prostate cancer (CRPC).

Estrogen Signaling

In addition to androgen, estrogen signaling also plays an important role in prostate growth and development [35-44]. Estrogen signaling is usually a balance between two opposing forces mediated by two estrogen receptors: estrogen receptor (ER) α and β. Both receptors belong to the nuclear receptor superfamily, which also includes androgen receptor, progesterone receptor, and glucocortocoid receptor among others [45, 46]. ERα was first discovered by Elwood Jensen in the late 1950s [47], while ERβ was discovered and cloned from rat prostate and ovary in 1996 [48]. Like other nuclear receptors, ERs can be divided in to six functionally distinct domains: the N-terminal (A/B) domains (including the ligand-independent activation AF1 domain), the DNA-binding (C) domain, the flexible hinge (D) domain and the C-terminal ligand-binding and ligand-dependent activation AF2 (E/F) domains. The most variable N-terminal domains share only 17% similarity between ERs. DNA-binding domain is the most conserved region, which has 97% amino acid homology between ERs. The ligand binding domain shares 56% identity,

while D domain has 36% and C-terminal F domain shows 18% homology between ERs (Figure I-2A).

Due to alternative splicing of the mRNA, different isoforms of each ER have been identified. Full length ERα and two smaller ERs, ERα46 and ERα36 are most widely studied. ERα46 lacks the first 173 N-terminal amino acids of the full-length ERα (66KDa), and was characterized as an inhibitor of ERα in osteoblasts [49, 50]. ERα36, lacking both transcriptional activation domains (AF1 and AF2), together with a unique 27 amino-acid sequence replacing the last 138 amino acids of full length ERα, primarily localizes at the plasma membrane and within cytoplasm, and plays a role in mediating non-genomic estrogen signaling [51-53]. ERβ has five known isoforms. All have novel C-terminals, and all variants, except for the full-length ERβ, lack the AF2 domain and cannot bind to estrogens (Figure I-2B).

Estrogen receptors are ligand-activated transcription factors. When bound to a ligand, the receptors will form either homo- or heterodimers, which then bind to estrogen response elements (EREs) on the DNA, and recruit co-regulators to regulate gene expression [54]. In addition to estrogens, ERs are also found to bind a variety of phytoestrogens, xenoestrogens, estrogen-disrupting chemicals and cholesterol derivatives called oxysterols [55-61], among which 27-hydroxycholesterol was shown to bind to an

allosteric binding site on ERβ in our lab [62].

The balance between ERα and ERβ is important for normal tissue growth, and the loss of either receptor can lead to different phenotypes. ERα is proposed to be involved in inflammatory and proliferative processes while ERβ is considered to be anti-inflammatory, anti-proliferative and increase apoptosis[37, 43, 63]. In neonatal DES treated mice, inflammatory cell infiltrate in the ventral and dorsolateral prostate lobes was observed with aging. ERβ knockout mice exhibited similar prostate response to the wild type (WT) mice, while in contrast, ERα knockout mice showed no response to DES, suggesting that estrogen induces inflammation through ERα[64]. Another group also reported anti-inflammatory effects of ERβ by showing 89% of ERβKO mice processing T cell infiltration compared to WT control, of which only 50% mice had inflammatory cells present [65]. In normal prostates and low-grade prostate cancer, ERβ is the predominant receptor found in the epithelial cells while ERα is present at very low concentrations in the stromal cells. However, in prostate cancer, ERα's expression is elevated and is found both in the stroma and epithelium and ERβ is almost completely lost in some prostate cancer samples [43, 66-74]. Our lab has shown that in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, knocking out ERα resulted in almost non-existent advanced cancer while knocking out ERβ resulted in an almost

doubling incidence of advanced tumors [43]. Therefore, ERβ can be a promising new target for prostate cancer treatment.

3,3'-Diindolylmethane (DIM)

DIM is the acid-derived dimer of indole-3-carbinol (I3C), which can be found in many cruciferous vegetables such as broccoli, kale and cabbage, via the mother compound glucobrassicin. When raw cruciferous vegetables are ingested, glucobrassicin is exposed to myrosinase, which is stored in the vegetables too. Myrosinase then catalyzes the hydrolysis of glucobrassicin and generates I3C. In the stomach, I3C rapidly turns into DIM (dimer), 5,11-dihydroindolo-[3,2-b] carbazole, and a cyclic triindole (trimer) via acid-catalyzed condensation (Figure I-3). When vegetables are cooked, myrosinase is inactivated thus the breakdown of glucobrassicin is prevented. Though I3C can still be generated due to myrosinase activity from colonic bacteria [75], DIM will not form in the alkaline environment of the intestine.

Biological functions of DIM

DIM has been shown to have many functions and interact with several pathways, such as the estrogen-signaling pathway, aryl hydrocarbon receptor pathway, androgen receptor-signaling pathway, Nrf2 pathway, NF-κB pathway, anti-angiogenesis effects, inducing cell apoptosis, causing G1/S arrest in cell cycle, etc. Figure I-4 summarizes the

biological functions of DIM. This suggests DIM is a multi-target drug which can inhibit cancer through multiple pathways. This can be a good thing, as multiple pathways could potentially work synergistically to achieve a greater effect. In addition, It is known that cancer always develops resistance to a new drug after prolonged exposure. Thus, when hit through multiple pathways, it will likely be harder for cancer cells to develop drug resistance, since a multi-target drug can still work through other pathways once one quits working. However, another hypothesis is that DIM really works mainly though one pathway. Because of the overwhelming amount of work done on these pathways and because our focus is on estrogen, we will discuss only those aspects of DIM functional pathways that relate to ERs. The biology inside a cell is very complicated. Every gene, protein, pathway is connected to each other and does not work in isolation. It's possible that all the effects previously shown by DIM are actually downstream connections to an unknown pathway. Thus, it is important to understand the primary mechanisms though which DIM works. This knowledge can provide us the information/ideas for next generation drug design.

1. Aryl hydrocarbon receptor (AhR) pathway

DIM is reported to bind to AhR with a Kd of 90nM in a competitive binding assay with [2-^{125}I] 7,8-dibromodibenzo-*p*-dioxin. [76, 77]. Ligand binding allows AhR to translocate into the nucleus and form a complex with the AhR nuclear translocator (Arnt). The

AhR/Arnt complex then binds to gene promoters, which include xenobiotic response elements (XRE), and induces downstream gene expression such as cytochrome P450 (CYP) enzymes. Microarray analysis of gene expression profiling showed CYP1A1 and CYP1B1 mRNA expressions were upregulated in DIM-treated both the human prostate cancer PC3 cell line and the human breast cancer MCF7 cell line, indicating DIM activated AhR in those cells[78, 79]. Some groups also reported DIM to be partial antagonist because of its inhibition of TCDD's activation of AhR in a human breast cancer cell line [80, 81].

2. Estrogen signaling pathway

DIM modulates estrogen signaling via two aspects: regulating estrogen synthesis and metabolism, as well as regulating estrogen receptor activity. CYP19 (aromatase) plays an important role in estrogen synthesis, which catalyzes the final step of androgen conversion to estrogen (Figure I-5). DIM was shown to decrease aromatase expression in estrogen-dependent MCF7 breast cancer cells, whereas in DIM-treated, estrogen-negative MDA-MB-231 cells, aromatase expression was increased [82]. In addition, another group also reported that DIM increased aromatase expression in human adrenocortical carcinoma cells [83].

Above we described how DIM could increase CYP1A1, CYP1B1 mRNA expression [78, 79, 83]. CYP1A1 and CYP1B1 are the enzymes catalyzing oxidative metabolism of estrogens. Products of these two enzymes in the cell are 2-hydroxy-estradiol/estrone (2OHE2/1) and 4-hydroxy-estradiol/estrone (4OHE2/1). In addition, endogenous estrogens can also be metabolized to 16α-hydroxy-estradiol/estrone (16αOHE2/1). 2OHE2/1 is considered to be a good estrogen with weaker estrogenic activity and was shown to be non-carcinogenic as well as inhibiting cell proliferation [84-87]. In contrast, 16αOHE2/1 is highly estrogenic and was found to stimulate cell proliferation[85, 88, 89]. Epidemiological studies also showed increased prostate cancer risk associated with elevated urinary 16αOHE1, reduced urinary 2OHE1 and lower ratio of 2OHE1 to 16αOHE (2:16OHE1) [90, 91]. In clinical trials, increased urinary 2:16OHE1 was found in DIM-treated group compared to the control, suggesting a possible mechanism for DIM's protective effects, which may be through shifting the metabolism of estrogen to "good" 2OH2/1 away from "bad" 16αOH2/1 [92, 93].

DIM was reported to activate both nuclear estrogen receptors' activities without competing with E2 at its binding site in a radio-labeled E2 competition assay [94-98]. However, the mechanism, through which DIM activates estrogen receptors without binding to the receptors, is not clear. One group reported that silencing of SRC-2 could inhibit the activation of ERβ-targeted keratin 19, NKG2E, and CECR6 genes by DIM,

suggesting DIM is activating ERβ by selectively recruiting ERβ coactivators [98]. Other groups also reported protein kinase A (PKA) inhibitor H89 ablated DIM's activation of ER, indicating PKA's involvement in this activation [95-97]. Previously, we have discussed that DIM may be a weak agonist of AhR. When AhR is activated, it translocates into the nucleus where it forms a complex with Arnt. This heterodimer complex can directly associate with ERα and ERβ, recruiting the co-activator p300, which has been reported to result in ligand-independent activation of estrogen receptors [99]. Finally, Our lab has found a second binding site in ERβ, which regulate ERβ's activity allosterically [62]. Our hypothesis is that DIM binds to ER at the allosteric binding site and activates estrogen receptor activities.

3. Androgen receptor (AR) signaling pathway

DIM is reported to be an androgen receptor antagonist, which inhibits AR's translocation into nucleus, thus reducing the expression of AR regulated genes, such as PSA[100]. Additinally, DIM was also shown to compete with DHT in a ^{3}H-DHT competitive binding assay with whole cell extracts and acted as a strong competitive inhibitor of DHT binding to AR [100]. In AR-positive and hormone sensitive LNCaP prostate cancer cells, B-DIM, a formulated DIM which has pure DIM microencapsulated in particles complexed with a vitamin E derivative and phospholipids to allow absorption from our intestinal track, downregulated the expression of AR [101]. The anti-androgenic effect of

DIM was also observed in a clinical study with prostate cancer patients, exhibiting AR nuclear exclusion and decreased concentration of PSA [102]. In addition, two groups reported DIM regulating AR signaling through other mechanisms, for example, by regulating micro-RNAs, which play a role in controlling AR splice variant expression and enzalutatmide resistance, or by epigenetic modulation of AR-dependent genes [103, 104], and so on.

4. Nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent pathway

Nrf2 is a transcription factor that plays an important role in regulating cytoprotective responses to oxidative stress. Nrf2 binds to the repressor protein Kelch-like ECH-associated protein 1 (Keap1) in the cytosol, which anchors its position in cytoplasm and promotes its degradation by ubiquitination. Oxidative or electrophilic stress causes conformational change of Keap1, thus releasing Nrf2 from Keap1. Free Nrf2 then translocates into nucleus, forms a complex with small Maf proteins, and finally binds to the antioxidant response element (ARE) in the promoter regions of target genes. Activation of Nrf2 induces expression of many antioxidant/detoxifying phase II enzymes such as glutathione S-transferases (GSTs), thioredoxin, NAD(P)H quinone oxidoreductase 1 (NQO-1), and heme oxygenase 1 (HO-1). DIM upregulates expressions of Nrf2 as well as GST, NQO-1 and HO-1 through Nrf2/ARE dependent pathway in multiple cell lines and also in *in vivo* mice studies [105-113]. In both TRAMP mice and

in vitro in the TRAMPC1 cell line, repressed expressions of Nrf2 and its target genes were found due to hypermethylation of CpGs in the promotor region of Nrf2 [109]. DIM suppresses DNA methyltransferases (DNMT) and histone deacetylases (HDAC) expressions, promotes the demethylation of Nrf2 gene promoter, and thus restores the expression of Nrf2 and its downstream genes, which further prevents prostate cancer development in TRAMP mice [109]. Our lab previously reported a cross talk between ERα and Nrf2 pathways. E2 activated ERα was able to bind to Nrf2 and inhibit its downstream gene expression [114]. Another group also reported that an anti-estrogen, such as tamoxifen, bound ERβ was able to form a complex with Nrf2 and then stimulated the expression of genes regulated by ARE [115].

5. Anti-inflammation through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway

Chronic inflammation has been shown to increase the risk of prostate cancer by promoting cell proliferation, angiogenesis and inhibiting apoptosis [116-120]. NF-κB is a transcription factor that regulates genes involved in immune response. In a mouse skin model, ear edema was induced by applying 12-O-tetradecanoylphorbol-13-acetate (TPA) to induce inflammation. DIM inhibited ear edema formation via inhibiting the TPA-induced NF-κB target genes cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) [121]. Besides, DIM also inhibited NF-κB's DNA binding activity as

well as reduced the TPA-increased IkappaB kinase (IKK), which includes activation of NF-κB [121]. This suggests that DIM exhibits anti-inflammation effects, which may contribute to its antitumor properties in prostate cancer. Estrogen receptors are also found to affect inflammation, with ERα promoting inflammation and ERβ having anti-inflammatory effects [64, 65]. Many studies have shown the inverse correlation between ER and NF-κB [122-125]. In high grade prostate cancer, loss of ERβ increased expression of IKKβ though activation of NF-κB. This same inverse correlation was also observed in the prostate of ERβKO mice [126].

Cholesterol, statins and prostate cancer

There has been a long-term interest in the literature in regard to the role of cholesterol in prostate cancer, especially when comparing cancer incidence worldwide. The United States and other developed countries, in which people usually eat a high fat western-style diet, have a much higher risk of developing prostate cancer, when compared with other Asian countries, such as China and Japan [127]. A majority of epidemiological studies have established a positive association between total cholesterol and overall or advanced prostate cancer incidence[128-130] . However, this conclusion remains controversial. Recently, serval epidemiological studies showed that hypercholesterolemia did not associate with overall or high-grade prostate cancer incidence [131]. In fact, people with

low sereum cholesterol had a high risk of prostate cancer or biochemical recurrence of prostate cancer after radical prostatectomy [132, 133].

Statins are inhibitors of HMGCoA reductase, a rate limiting enzyme in the cholesterol synthesis pathway. They bind to HMGCoA reductase with a lower Kd than its natural ligand, HMGCoA. This binding prevents the enzyme from forming a functional structure, thus reducing mevalonate's concentration within the cell [134]. Enzymatic studies indicate that Ki for most statins are 5–44 nM while the Km of HMG-CoA is 4 μM, thus statins are potent inhibitors of this enzyme [135-137]. HMGCoA reductase catalyzes cholesterol synthesis, thus inhibiting this enzyme reduces cholesterol levels and increases LDL-receptor expression.

Epidemiological evidence has suggested the potential benefit of statins, a class of cholesterol lowering drugs, to prevent prostate cancer or advanced prostate cancer [138-140]. Due to the physicochemical properties, statins can be divided into two categories: hydrophobic and hydrophilic. Among them, simvastatin and lovastatin are the most hydrophobic, with a lactone ring in their structure, while pravastatin is the most hydrophilic because of the open acid structure. Epidemiological studies have shown that simvastatin and pravastatin exhibit different abilities to inhibit cancer[141]. Generally, hydrophobic statins such as simvastatin can easily cross the lipid bilayer membrane by

passive diffusion, which means they will distribute to all tissues nonspecifically [142]. In contrast, hydrophilic statins can't cross the membrane freely and need an active transport system. Pravastatin is mainly taken up by the liver through organic anion transporting polypeptides (Oatps) [143, 144]. This may explain why there was a difference in effects between different statins.

General Overview

In Chapter II, we explored the mechanism of how DIM inhibited prostate cancer cell proliferation *in vitro*. We **hypothesize** that DIM inhibits prostate cancer through modifying estrogen receptor activity, especially ERβ's activity. We tested DIM's inhibitory effect on the growth of various cancer cell lines. We found that DIM stimulated both nuclear estrogen receptors' activities and the stimulations could be inhibited by ER antagonist ICI. We also performed ligand binding competition assays with ^{3}H-E2, indicating that DIM could not compete with E2 by binding to the canonical binding site. In the end, we ruled out the possibility that AhR mediated the activation of ER by DIM.

In Chapter III, the effect of DIM on advanced prostate cancer incidence was tested *in vivo* in our TRAMP mouse model. We **hypothesize** that DIM can inhibit PDC in TRAMP, dose dependently. We found a trendline of decreasing PDC along with increasing DIM

concentration in the diet. Though weight loss was found in the group of mice fed with the highest concentration of DIM, we were able to eliminate the possible positive correlation between mouse body weight and PDC incidence. In addition, we performed three independent experiments on the ERKO mice and successfully explained that the weight loss was independent of ER function.

Chapter IV summarizes my research and suggests future study directions.

In Appendix-I, we discussed about the effect of dietary cholesterol on prostate cancer incidence in the TRAMP mouse prostate cancer model.

Results of Pectasol-C in TRAMP mice were shown in Appendix-II.

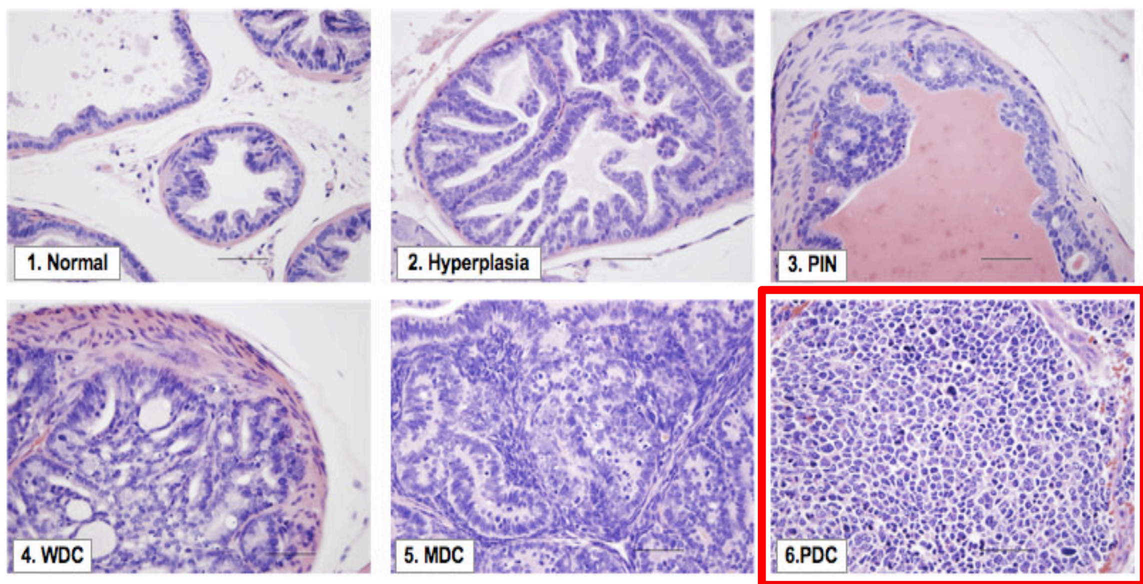
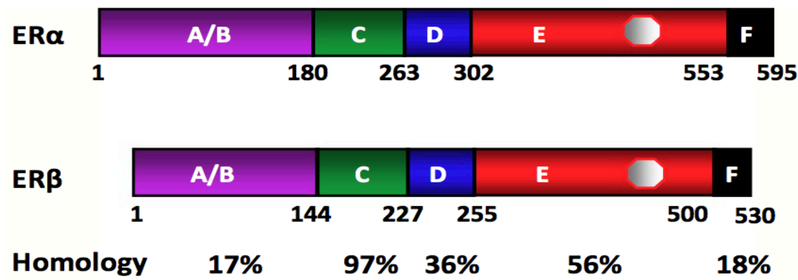


Figure I- 1: Histology Sections of TRAMP Tumors.

1. Normal morphology, 2. Hyperplasia, 3. Prostatic Intraepithelial Neoplasia, 4. Well-differentiated Carcinoma, 5. Moderately-differentiated carcinoma, 6. Poorly-differentiated carcinoma. Images provided by Dr. Cynthia Besch-Williford.

A)



B)

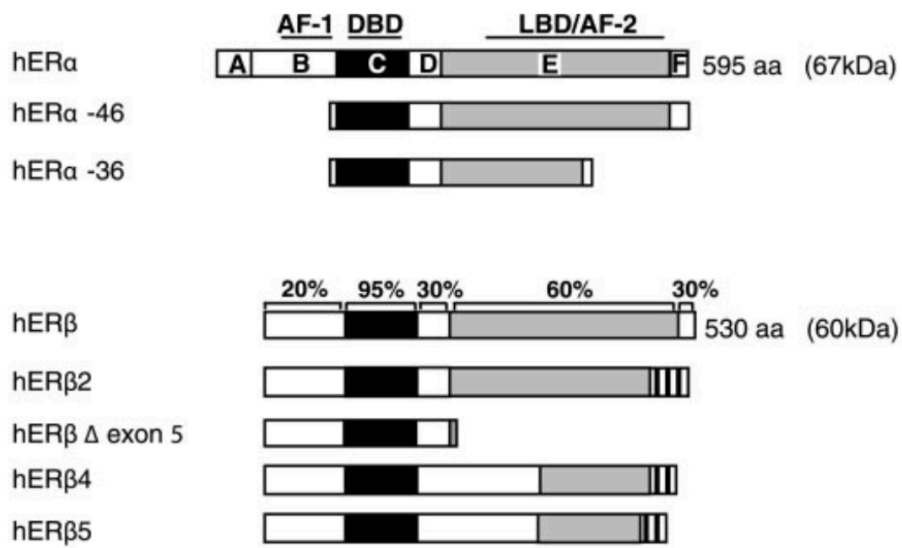


Figure I- 2: Schematic of the estrogen receptor(ER)α and ERβ structural regions and isoforms.

Original figure by A) Muyan, et al [145], B) Gustafsson, et al [52]

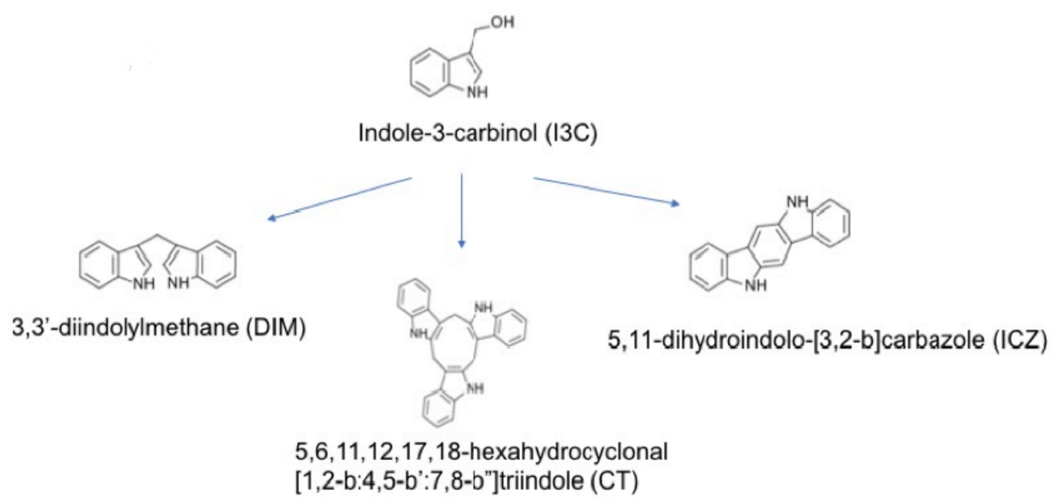


Figure I- 3: Condensation derivatives of Indole-3- Carbinol.

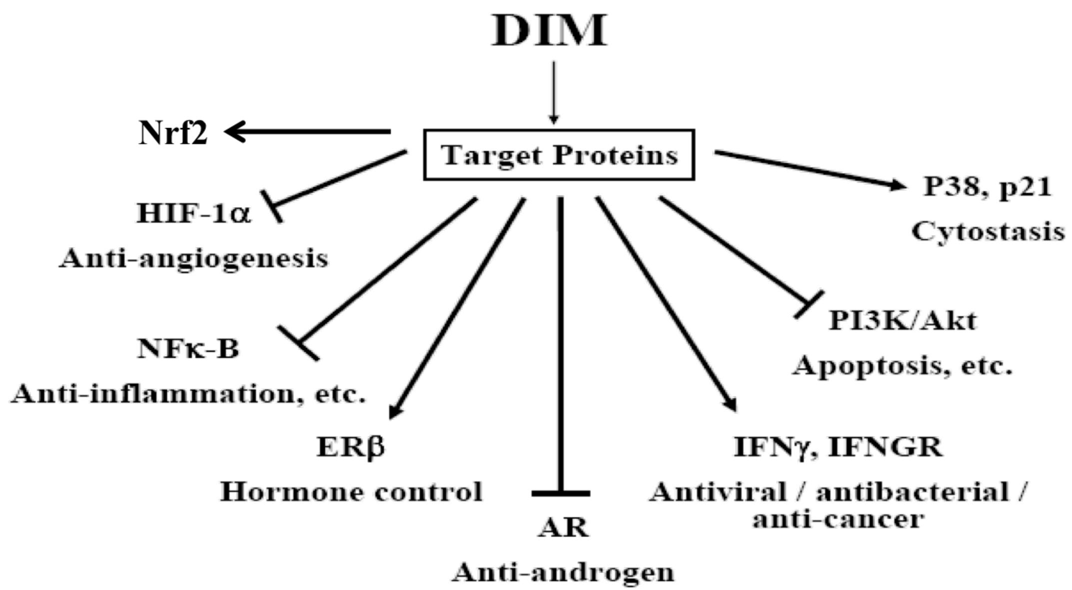


Figure I- 4: Biological functions of DIM.

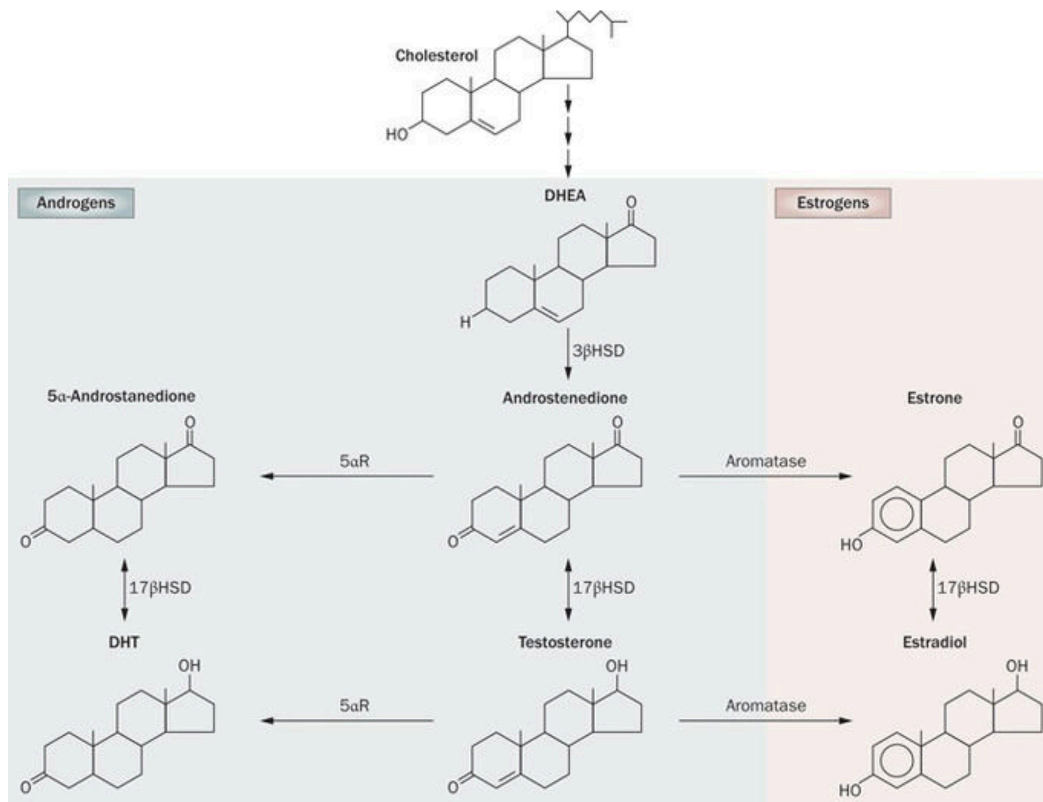


Figure I- 5: Biosynthesis of estrogen and androgen.

Original figure by Clement K. M. Ho, et al [146]

CHAPTER II

DIM INHIBITING CANCER CELL GROWTH AND MODULATING ESTROGEN RECEPTOR ACTIVITY IN VITRO

Overview

3,3'-Diindolylmethane (DIM) is a compound derived from many cruciferous vegetables, including broccoli, cabbage, Brussels sprouts and cauliflower. DIM is reported to exhibit anti-cancer effect in several *in vitro* and *in vivo* cancer models. DIM has been reported to interact with many pathways, including the estrogen receptor (ER) pathway. However, the role of estrogen receptor in the anti- cancer effect of DIM is still not clear. Here we hypothesize that ERβ plays an important role in DIM' s inhibitory effect on cancer. We found that DIM stimulated both nuclear estrogen receptors activities in a luciferase assay performed in U2OS bone and C4-12-5 breast cancer cell lines. *In vitro* tritiated 17β-estradiol (^{3}H-E2) competitive binding assay showed that DIM did not compete with E2 in binding to ERβ. By testing DIM's effect on cell growth in cell lines with different ER status, we found that ERβ was necessary for DIM's inhibition of cancer cell growth. In conclusion, our results suggest that DIM inhibits prostate cancer through an estrogen receptor signaling pathway.

Introduction

Natural compounds are well known to be a good source of lead compound for drug discovery and development. The use of herbal medicine to treat human disease can date back to thousands of years. Due to their time-tested safety and efficacy, modern drug researchers gain great insights by studying plant-derived compounds. Many modern

studies have been performed to test the efficacy, as well as the mechanisms of action, of these plant-derived compounds [147, 148].

3,3'-Diindolylmethane (DIM) is one of those plant-derived compounds. DIM is the acid-derived dimer of Indole-3-carbinol (I3C), which is present at relative high levels in cruciferous vegetables. Epidemiological studies have demonstrated that intake of cruciferous vegetables, such as broccoli, kale and cabbage will reduce the risk of advanced prostate cancer [149-151]. I3C itself also has been shown to inhibit prostate cancer [152, 153]. However, I3C is not stable at low pH and will spontaneously form DIM in the stomach. Thus, DIM is considered to be the bioactive product and be responsible for the anti-cancer effect of I3C in the body.

Estrogen Receptor alpha (ERα) and Estrogen Receptor beta (ERβ) are the two nuclear estrogen receptors, which bind to the Estrogen Response Elements (EREs) on the DNA and regulate gene transcription after binding and activation by the ligands [47, 48, 154-158]. ERα and ERβ usually have opposite functions during development, with ERα involved in proliferative process, while ERβ showing an anti-proliferative effect and increasing apoptosis [37, 43]. Previous studies from our lab showed that knocking out ERβ resulted in almost double the incidence of PDC in the TRAMP mouse model, while knocking out ERα resulted in almost no advanced prostate cancer [43]. This indicates

that ERβ prohibits prostate cancer while ERα promotes cancer.

DIM is reported to have interactions with several different pathways, such as the aryl hydrocarbon receptor pathway, androgen receptor signaling pathway, Nrf2 pathway, NF-κB pathway, and the most interesting to our lab, the estrogen receptor signaling pathway, suggesting DIM is a multi-target drug [79, 81, 98, 100, 107, 109, 121]. However, the primary mechanism through which DIM works to inhibit cancer is still not clear. In this study, we hypothesized that DIM inhibited prostate cancer through modulating estrogen receptor, especially the estrogen receptor beta-signaling pathway. To test our hypothesis, we tested DIM in a transcriptional luciferase reporter assay and demonstrated that DIM stimulated both ERα and ERβ transcriptional activities. Results from growth assays in cell lines with different ER status supported our hypothesis that ERβ mediated DIM's inhibition of cancer cell growth.

Materials and Methods

Reagents

17β-estradiol (E2) was purchased from Sigma Aldrich (St. Louis, MO). DIM was purchased from Sigma Aldrich (St. Louis, MO). Doxycycline was purchased from Sigma Aldrich (St. Louis, MO). ICI 182,780 was a gift from Dr.Rex Hess (UI-UC). For competitive binding assay, tritiated 17β-estradiol (^{3}H-E2) was purchased from American

Radiolabeled Chemicals (St. Louis, MO) and Perkin Elmer (Waltham, MA). Purified full length recombinant ERβ (P2466) proteins (Panvera) were purchased from Invitrogen/Life Technologies (Grand Island, NY).

Cell Culture

TRAMP-C2, DU145, LNCap and MCF7 cells were obtained from the American Type Culture Collection (ATCC). U2OS cells were provided by Dr. Hansjorg Rindt (University of Missouri, Columbia, MO). TRAMP-C2 cells were maintained in Dubelcco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (GE Healthcare Life Sciences, Logan, Utah). DU145, LNCap cells were cultured in RPMI 1640 media (Invitrogen, Grand Island, NY) with 10% FBS. U2OS cells were cultured in phenol red-free DMEM/F-12 media (Invitrogen, Grand Island, NY) containing 5% FBS. MCF7 cells were maintained in DMEM (Invitrogen, Grand Island, NY) containing 10% FBS. ER negative MCF-7 cells, C4-12-5, were maintained in complete medium consisting of phenol red-free Eagle's Minimal Essential Medium (MEM) (Invitrogen, Grand Island, NY) supplemented with 5% charcoal stripped FBS.

RNA isolation and Reverse Transcriptase (RT) PCR

Total RNA was isolated from TRAMP-C2 cells using the RNeasy kit (Qiagen, Venlo, Netherlands). RNA concentration was determined using the ND 1000 Spectrophotometer

v3.1 (NanoDrop Technologies, Wilmington, DE). 1000ng of total RNA was used to create cDNA libraries using Superscript III Reverse Transciptase with random primers and oligodT (Invitrogen, Grand Island, NY). PCR was used to determine the presence of ER mRNA and was performed for 30 cycles at 95°C for 30 seconds, 65°C for 1 minute, 72°C for 25 seconds, followed by the final extension step of 72°C for 5 minutes. Primers used for detecting mouse ERα: fERα- GACCAGATGGTCAGTGCCTT (1139-1158); rERα- ACTCGAGAAGGTGGACCTGA. (1343-1324). Primer Sequences for mouse ERβ: fERβ- GTAGAGAGCCGTCACGAATACT (166-187); rERβ- GGTTCTGCATAGAGAAGCGATG (362-341).

Growth Assay

TRAMPC2, DU145, LNCap and MCF7 cells were pre-incubated in 24-well/96-well plates in a phenol-red free, charcoal-stripped serum media for one day before treatment. After treatment, cells were lysed with 1N NaOH after 0hr, 24hr, 48hr, 72hr and 96hr of additional growth. Total protein concentration was measured with the Bio-Rad DC kit (Bio-Rad, Hercules, CA) by measuring absorbance at 750nm. Total protein had been previously shown to correlate with the thymidine incorporation assay in prostate cancer cells, as a reliable measure for cell growth [159].

Luciferase Assay

U2OS cells were pre-incubated in phenol red-free DMEM/F-12 media supplemented with 5% charcoal-stripped FBS for 24hrs. C4-12-5, were maintained in phenol red-free MEM supplemented with 5% charcoal stripped FBS. Cells were transfected with the vitellogenin ERE luciferase reporter plasmid, the SV40-Renilla control vector, and either the ERα or ERβ expression vector using Fugene HD (Promega, Madison, WI) for 18 hours. Transfected cells were seeded to 96-well plates and then followed by treatment for 24 hours. After that, cells were lysed using passive lysis buffer (Promega, Madison, WI) and transferred to assay plates. Luciferase and Renilla activities were measured by Biotek Synergy 2 plate reader (Winooski, VT) using the Dual-luciferase assay kit (Promega, Madison, WI).

Competitive binding assays

Purified human ERβ was diluted in a buffer containing 10mM Tris, 1mM EDTA, 10% v/v glycerol, 1mM EGTA, 1mM PMSF, 1mM DTT and 0.01% w/v BSA, at a final concentration of 0.45nM. 3nM ^{3}H-E2 was added to the protein in the absence presence of various concentrations of unlabeled compound and the mixture was incubated overnight at 4°C to achieve binding equilibrium. To separate the bound and free ligand, the mixture was added with 1% w/v dextran coated charcoal (DCC) and incubated for 15 minutes, and then centrifuged for 10 minutes. The supernatants were collected and finally measured by a Beckman LS 6500 scintillation counter (Beckman, Fullerton, CA) with

addition of Scintisafe 30% scintillation cocktail (Fisher Sci., Fairlawn, NJ).

Results

DIM stimulates both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) transcriptional activities in C4-12-5 breast cancer cells

Because of the anti-cancer effect of ERβ in TRAMP mice [43], the long-term goal in our lab is to find a compound that could specifically stimulate ERβ or inhibit ERα and prevent prostate cancer. DIM is reported by Vivar, O. at.el to be a selective ERβ agonist with no activation of ERα at all [98]. We were very excited to find this compound and decided to verify its selectivity in our *in vitro* luciferase transcription system. Since there was no ER-negative prostate cancer cells, we first tested DIM in the MCF7 derived ER-negative C4-12-5 breast cancer cell line. Cells were cotransfected with the vitellogenin estrogen response element (vERE) driven luciferase reporter, the SV40-Renilla control vector, and ER expression vectors. The transcriptional activity was determined by measuring luciferase expression normalized for renilla expression.

Before the experiment, we checked and verified there was no background ER present in the cells by cotransfecting the pcDNA3.1 control plasmid instead of ER expression plasmid. Adding E2 or DIM could not stimulate the luciferase expression when pcDNA3.1 was transfected (Figure II-1). Next, we cotransfected C4-12-5 cells with ERα

or ERβ expression plasmids and treated cells with 1-30uM DIM. DIM stimulated ERβ's transcriptional activity with an EC50 ~10uM. Adding ICI 182,780, the antagonist of ER, abolished the stimulation by DIM, indicating that this activation was mediated by ERβ (Figure II-2B). However, surprisingly, similar results were also seen when cells transfected with ERα, with an EC50 <10uM (Figure II-2A).

DIM stimulates both ERα and ERβ transcriptional activities in U2OS cells

Because of the discrepancy between our results and the previous report by Vivar, O. at.el that DIM is a ERβ selective agonist [98], which we initially thought might be a cell type specific effect, we decided to use the same cell line, Human Bone Osteosarcoma Epithelial Cells (U2OS), as in their paper. ERE luciferase assay with transient transfected pcDNA3.1, ERα or ERβ was performed the same way as described in C4-12-5 cells. No background ER activity was observed when U2OS cells were transfected with pcDNA3.1 (Figure II-3). Similar results were observed as we saw in C4-12-5 cells. DIM activated both ERα and ERβ transcriptional activities in a dose dependent manner. EC50 of ERα was ~3uM (Figure II-4A), while EC50 of ERβ was ~10uM (Figure II-4B). In addition, when both estrogen receptors were activated by E2, adding DIM did not affect the activation, with only a very slightly stimulation at the highest concentration of 30uM (Figure II-5).

In Vivar, O. et.al's paper, they used ERα/β stable transfected U2OS cell line for ERE luciferase assay. Doxycycline was used to induce the expression of estrogen receptors. In order to minimize any difference between our and their systems, we also tested DIM's estrogenic activity in the presence of doxycycline. Doxycycline alone did not stimulate either estrogen receptor. Adding doxycycline did not affect DIM's stimulation of both ERs neither (Figure II-6).

DIM did not compete with E2 in binding to ERβ

Besides its ability to activate ER's transcriptional activity, we also checked DIM's binding to ERβ through an *in vitro* competitive binding assay with radiolabeled E2 (^{3}H-E2). DIM did not show any competition with E2 in this assay (Figure II-7), suggesting DIM did not bind to the orthosteric binding site of ERβ.

CH223191 did not inhibit DIM's stimulation of ER transcriptional activity

DIM is reported to be a Selective Aryl Hydrocarbon Receptor Modulator (SAhRM) [76, 77, 160, 161]. Aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor. Following ligand binding, AhR will translocate into nucleus and dimerize with the AhR nuclear translocator (Arnt), which then binds to xenobiotic response elements (XREs) in the promoter or enhancer regions and regulates downstream gene expression. The AhR/Arnt complex can also work as co-regulator, recruiting unliganded ER and other

co-activator, and induce the ligand independent activation of ER transcription activity [99]. We decided to test whether AhR was involved in DIM's activation of ER.

We chose to use CH223191, an AhR specific antagonist with no affinity to ER. If AhR mediated DIM's ligand independent activation of ER, adding CH223191 would remove the stimulations by DIM in the ERE luciferase assay. However, this was not the case. Ch223191 alone did not show any activation of ER or inhibition of E2 activated ER in the ERE luciferase assay (Figure II-8A, B). Addition of CH223191 did not affect DIM's activation of ER neither, suggesting that the activation of ER transcriptional activity in U2OS cells was not through activation of AhR (Figure II-8C, D).

DIM inhibited the growth of ERβ positive cancer cell lines

We showed that DIM could activate both estrogen receptors. Since ERα and ERβ play opposing roles in cell growth-- ERα is proposed to be involved in proliferative process, while ERβ usually shows anti-proliferative effects and increases apoptosis [37, 43], we tested DIM for its effects on cell growth in five cell lines each with a different ER status. Prostate cancer cell lines LNCap and DU145 only express ERβ and not ERα [162]. Cells were treated with or without DIM at three concentrations: 10uM, 30uM and 60uM for 96h. DIM inhibited the growth of LNCap and DU145 at all three concentrations (Figure II-10A, B). Prostate cancer cell line PC3 expresses both ERα and ERβ [162]. The

inhibitory effects of DIM on cell growth were only observed at higher concentrations, while low concentration (10uM) of DIM showed no effects (Figure II-10C). In contrast, in MCF7 cell line, a human breast cancer cell line which only expresses ERα, 10uM DIM stimulates the cell growth (Figure II-10D). This is consistent with the observation from another group that low concentrations of DIM stimulated ERα and induced cell proliferation [96]. In addition, the MCF7 cells were more sensitive to DIM, with 60uM DIM showing cell toxicity. TRAMP-C2 is a mouse prostate cancer cell line derived from TRAMP mice that expresses both ERα and ERβ (Figure II-9). DIM inhibited the growth of TRAMP-C2 with IC50 >30uM (Figure II-10E). Taken together, our results suggested that ERβ might play an important role in mediating the inhibitory effect of DIM on cell growth.

Discussion

While our world is not flat, everything is always connected with each other. For a multi-target drug, like DIM, which seems to interact with so many pathways, it is always interesting to see whath the first interaction is, or determine the primary mechanism of how it works in a specific disease. Among those pathways, estrogen receptor signaling is always the one that catches the eye of our lab. Based on previous findings from our lab, knocking out ERβ in TRAMP mice greatly increased the incidence of PDC, while knocking out ERα protected against PDC [43]. We hypothesized that DIM inhibited

prostate cancer through activating ERβ, which is protective to cancer.

DIM was reported by Vivar et.al to be a selective ERβ agonist in U2OS, HeLa and MDA-MB-231 cell lines in the luciferase reporter assay and did not stimulate ERα at all [98]. Interestingly, we did find DIM activated both estrogen receptors in U2OS and C4-12-5 breast cancer cell lines. For the same cell line that we both had in hand, the U2OS cell line, we tried to repeat the published results, including using the same media, the same ERE promoter, adding doxycycline together with DIM, etc. Unfortunately, none of those helped us get the selectivity of ERβ by DIM. However, our data is in agreement with several other reports that DIM activates ERα in MCF7 breast cancer and Ishikawa cell lines either using a luciferase reporter assay or measuring endogenous ERα-regulated gene expression [95, 96].

On the other hand, DIM does not compete with E2 in a radio-labeled E2 competitive binding assay [97, 98] (Figure II-7). Explanation of this ligand-independent activation of DIM is still not clear. One explanation can be that DIM activates ER by recruiting ER coactivators. It is reported that silencing of SRC-2 can inhibit the activation ERβ targeted keratin 19, NKG2E, and CECR6 genes by DIM [98]. Moreover, DIM activated Arh forms a complex with Arnt, which can directly associate with ERα and ERβ, recruiting the co-activator p300 and activating estrogen receptors [99], though we tested the AhR

antagonist– CH223191 together with DIM in our system, and it did not affect the activation of ER by DIM (Figure II-8). Other groups also reported that activation of ER by DIM could be ablated by adding protein kinase A (PKA) inhibitor H89, indicating PKA 's involvement and possible ER phosphorylation during this activation [95-97]. Recently, our lab reported an alternate binding site present in ERβ, which can allosterically regulate ERβ's activity [62]. It is possible that DIM binds and activates ER from an alternate binding site but does not allosterically influence the binding affinity of E2 in the orthosteric site in an appreciable way (Figure II-7). Thus, DIM is not a competitive ligand with estradiol on estrogen receptors.

Because of the opposing biological functions of ERα and ERβ in cancer, we performed growth assays in cell lines with different ER subtypes to determine if estrogen receptor mediates the inhibitory effect of DIM on cancer cell lines. DIM only showed dose-dependent inhibitory effect in cell lines with ERβ present (Figure II-10A-C, E). In ERα-only MCF7 cells, DIM even showed a stimulation of growth at 10uM (Figure II-10D). Interestingly, at a higher 30uM DIM concentration, the growth stimulation was decreased. This suggests more than one pathway is regulated by DIM in the MCF-7 cell line.

Besides its activation of estrogen receptor activity, DIM may also function by regulating

the concentrations of ER within the cell. In the MCF7 cell line, 10uM DIM could reduce the ERα mRNA expression by almost 90% compared to controls [163]. Additionally, feeding male rats with DIM could reduce the mRNA expression of ERα in prostate gland [164]. On the other hand, DIM was reported to stimulate the expression of ERβ mRNA in hippocampal cells exposed to hypoxia [161]. Though in their paper, the increase in mRNA was not reflected in changing protein concentration, it still gives us clue of what may happen in the prostate. Thus, it is possible that DIM inhibits prostate cancer by increasing the cancer protective ERβ, while decreasing the cancer promotional ERα. It would be interesting to check the ER expression in the DIM TRAMP study in Chapter III to verify this hypothesis.

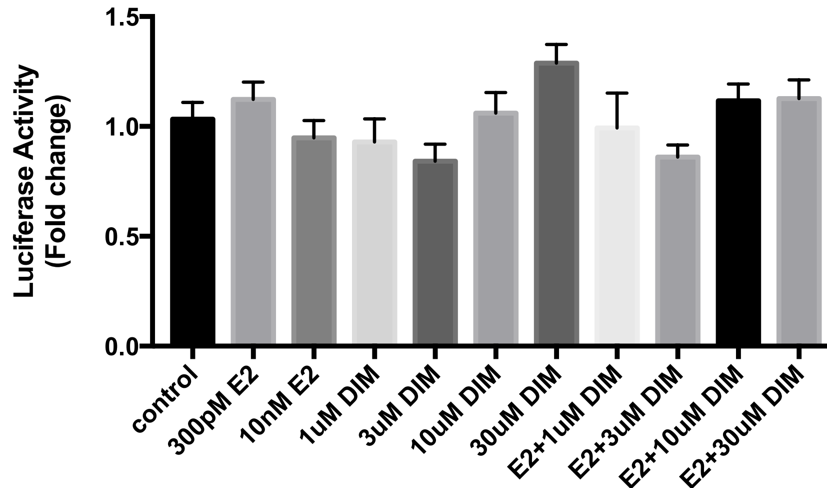


Figure II- 1: ERE luciferase assay with pcDNA 3.1 control plasmid in C4-12-5 cells. Error bars indicates S.E.M.

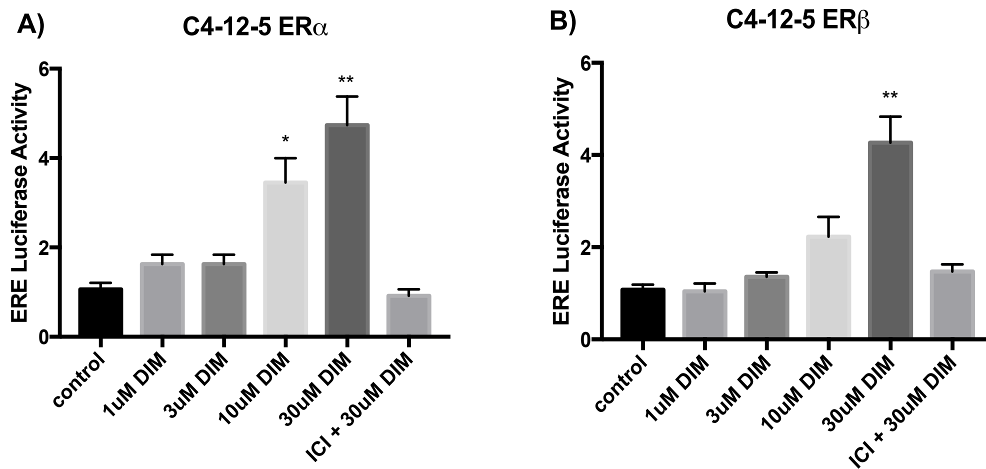


Figure II- 2: DIM stimulates both estrogen receptors in C4-12-5 cells

DIM stimulates ERa **A)** and ERβ **B)** transcriptional activities in an ERE luciferase assay in MCF7 derived ER negative C4-12-7 cells. Adding 100nM ICI 182,780 were able to abolish the stimulations caused by DIM. Error bars indicates S.E.M. * indicates $p < 0.01$, ** indicates $p < 0.001$ compared to control

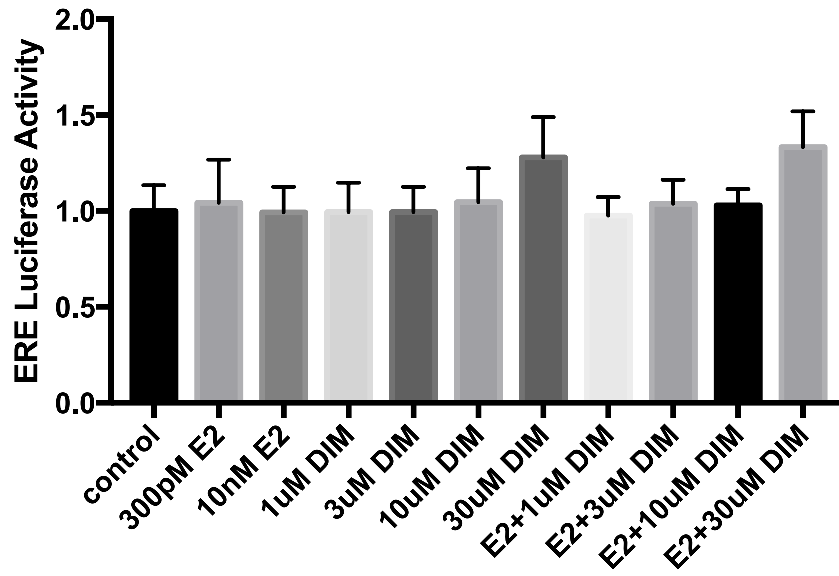


Figure II- 3: ERE luciferase assay with pcDNA 3.1 control plasmid in U2OS cells. Results are expressed as means ± SD.

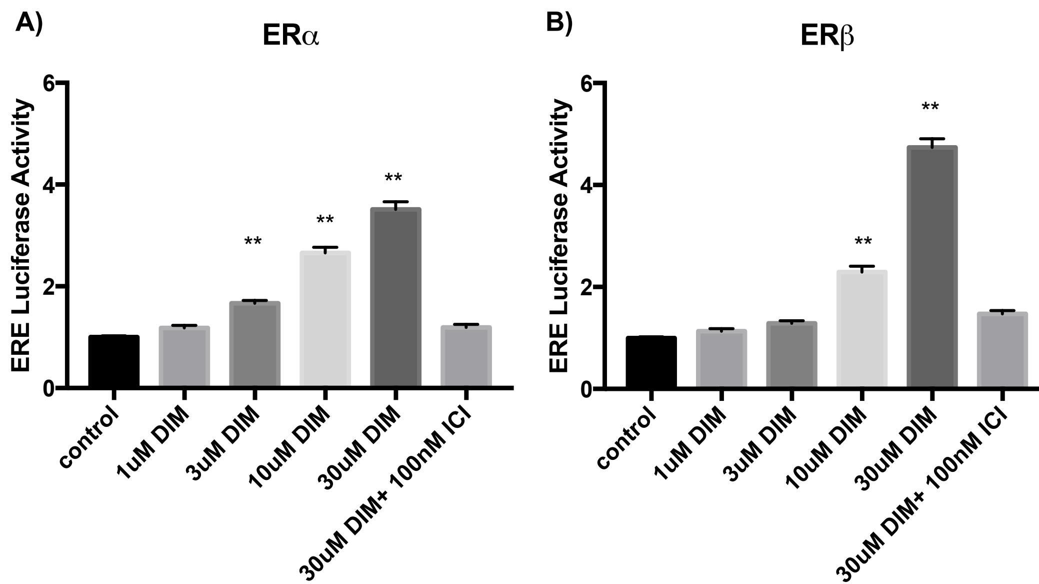


Figure II- 4: DIM stimulates both estrogen receptors in U2OS cells.

DIM stimulates ERa **A)** and ERβ **B)** transcriptional activities in an ERE luciferase assay in U2OS cell line. Error bars indicates S.E.M. ** indicates $p<0.001$ compared to control.

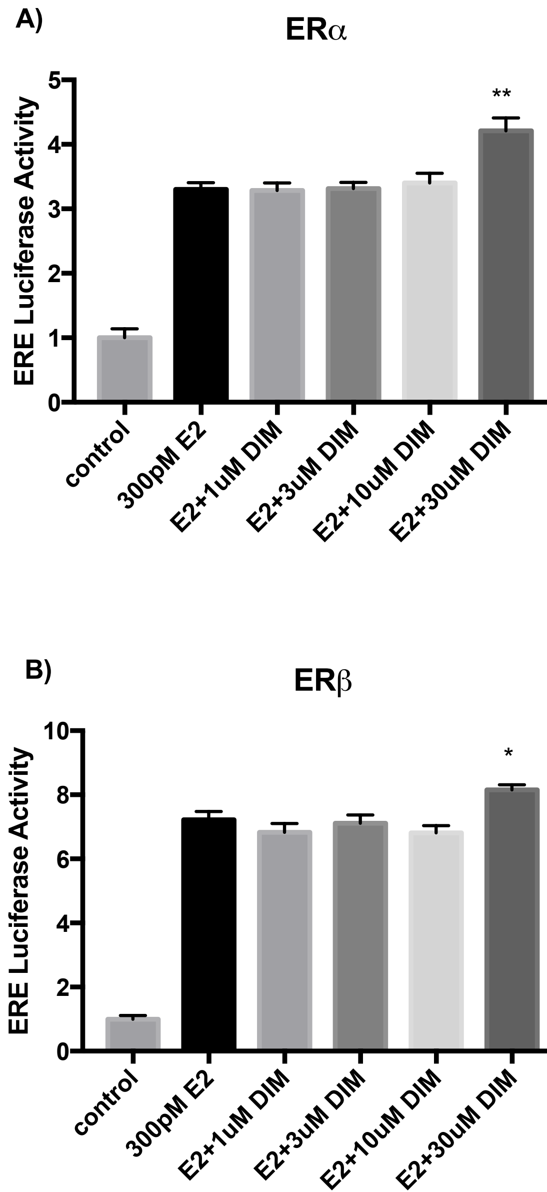


Figure II- 5: DIM did not affect E2's stimulations of estrogen receptor

ERE luciferase assay with **A)** ERα and **B)** ERβ in U2OS cell line. Error bars indicates S.E.M. * indicates $p<0.01$, ** indicates $p<0.001$ compared to 300pM E2.

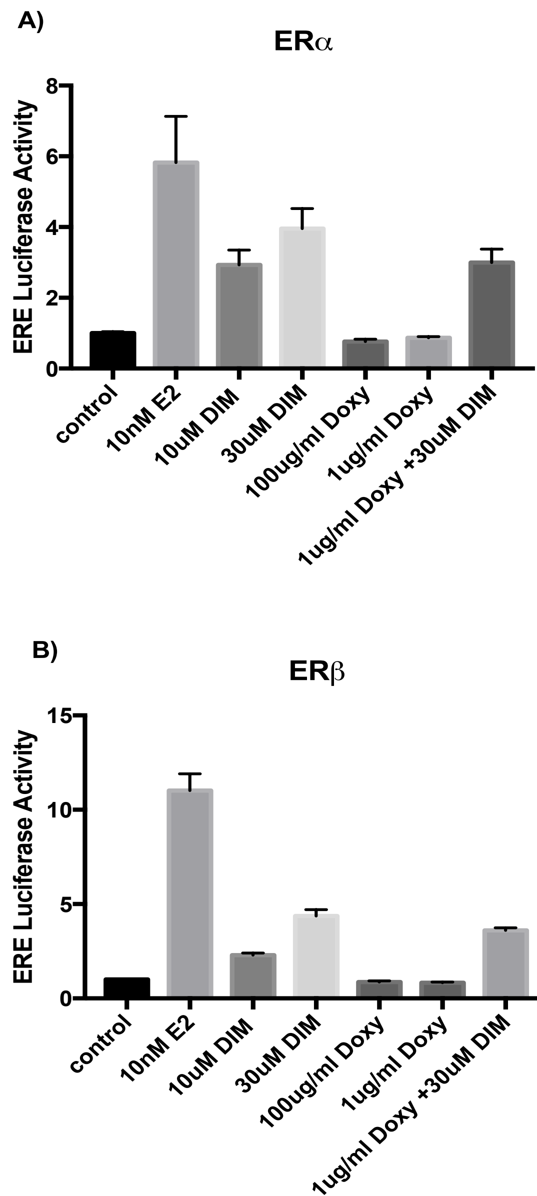


Figure II- 6: Doxycycline did not affect DIM's stimulation of estrogen receptor activity.

ERE luciferase assay with **A)** ERα and **B)** ERβ in U2OS cell line. Error bars indicates S.E.M.

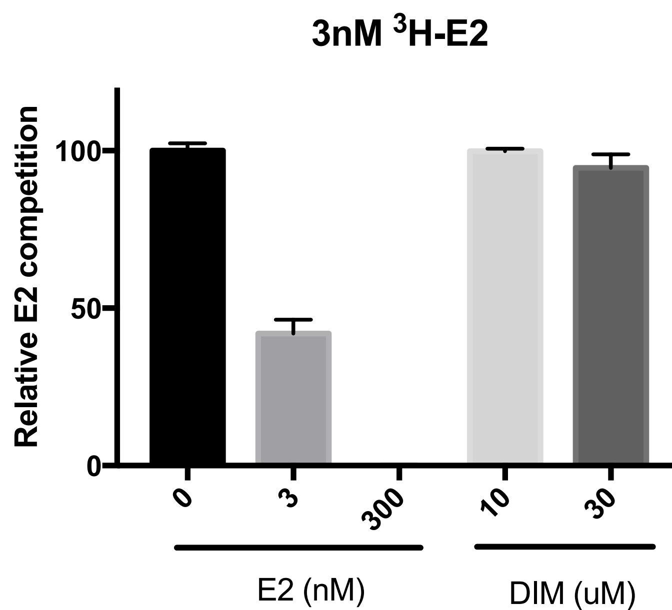


Figure II- 7:Competitive binding of DIM with human estrogen receptor hERβ.

DIM does not compete with tritiated estradiol (^{3}H-E2) (3nM) for binding to hERβ but in the positive control the unlabeled E2 does. Error bars indicates S.E.M.

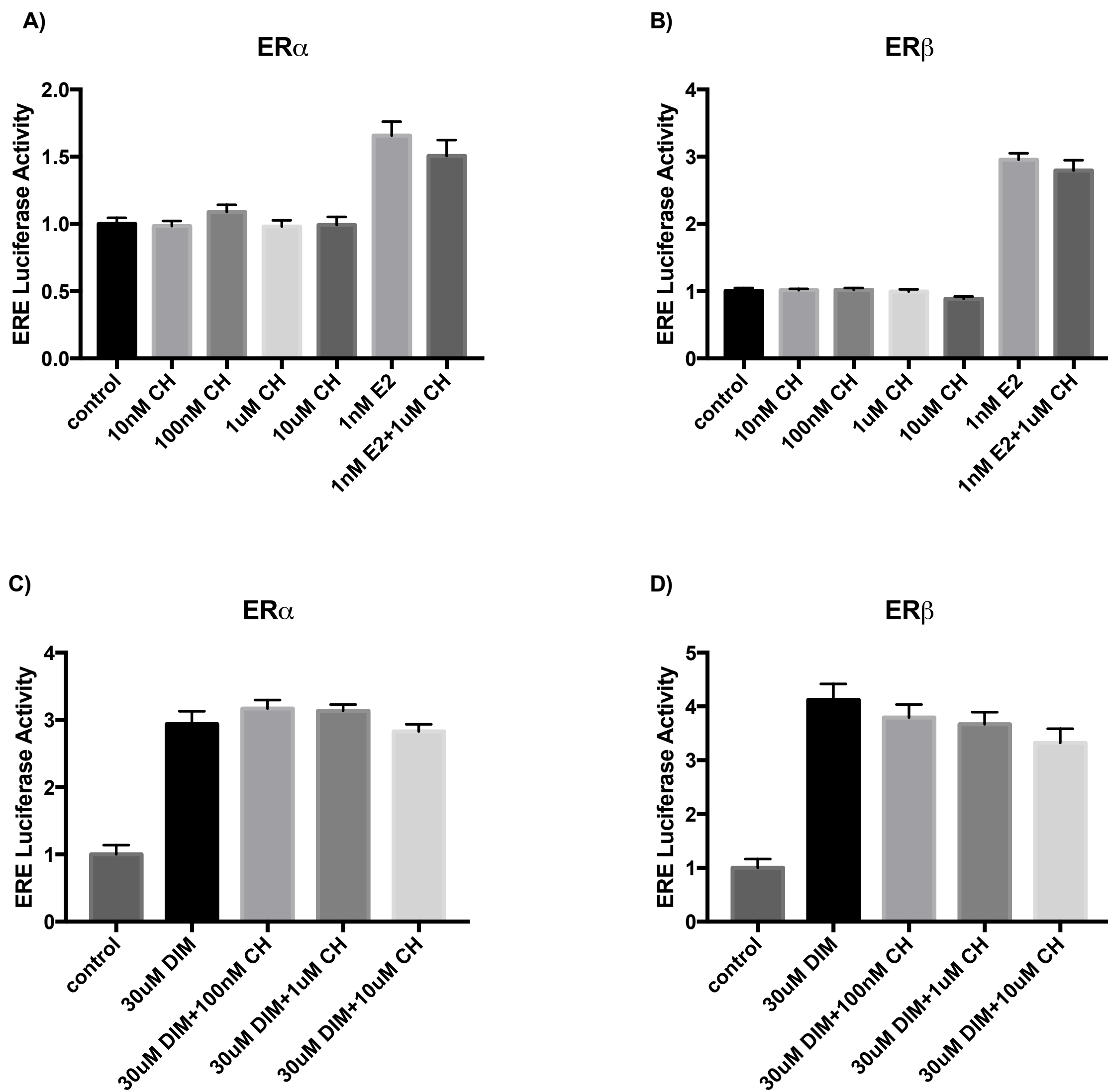


Figure II- 8: CH223191 did not affect DIM's stimulation of estrogen receptor activity.

ERE luciferase assay performed in U2OS cells. Error bars indicates S.E.M.

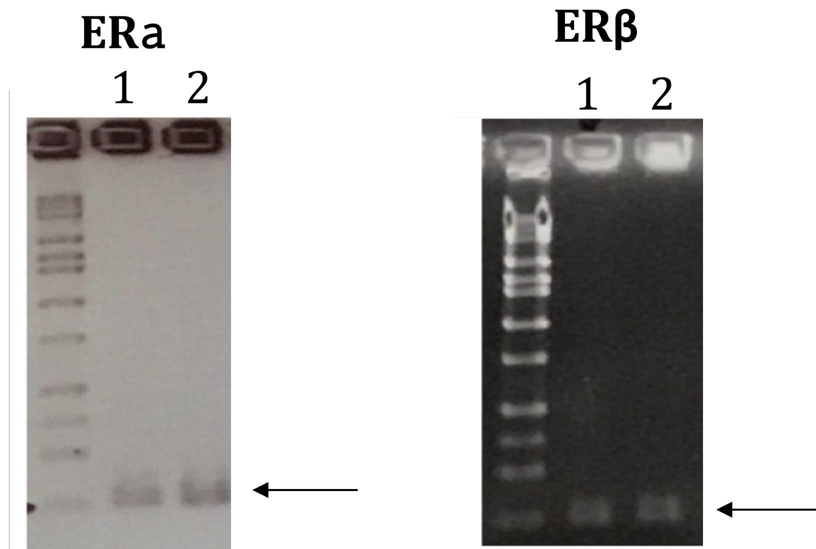


Figure II- 9: RT-PCR analysis of ERα and ERβ expression in TRAMP-C2 cell line.

Lane 1 and 2 are duplicates of amplified cDNA libraries made from 2 independent mRNA samples. Arrows indicate presence of 205bp ERa and 197bp ERβ bands

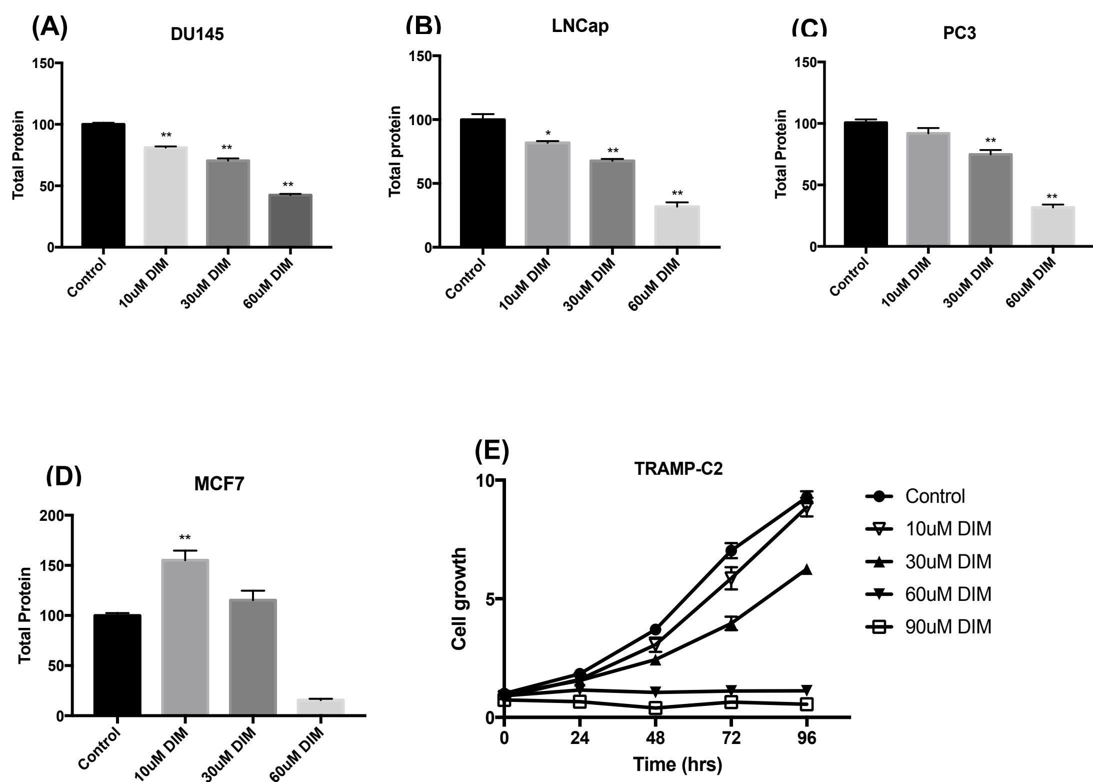


Figure II- 10: Effects of DIM on cell growth.

A) Du145, **B)** LNCaP, **C)** PC3 and **D)** MCF7 cells were plated in 24-well plate at 50% confluence. Cells were then treated with 0-60uM DIM for 96 hrs. The total cellular protein concentration was measured and normalized to the control of each cell line. **E)** TRAMP-C2 cells were plated in 24-well plate and treated with 0-90uM DIM. The total cellular protein concentrations were determined every 24hrs for total 96hrs. Protein concentrations were normalized to the control at 0h. Error bars indicates S.E.M. * indicates p< 0.01, ** indicates p<0.001 compared to control

CHAPTER III

DIM INHIBITS ADVANCED PROSTATE CANCER DEVELOPMENT IN TRAMP MICE

Overview

3,3'-Diindolylmethane (DIM) is an acid-derived dimer of indole-3-carbinol, found in many cruciferous vegetables, such as broccoli, and has been shown to inhibit prostate cancer (PCa) in several *in vitro* and *in vivo* models at high concentrations. We **hypothesize** that DIM will inhibit advanced PCa at lower concentrations and that DIM inhibits advanced PCa via stimulation of ERβ. To further study the effects of DIM on inhibiting advanced PCa development, we used FVB/C57 TRAMP (**TR**ansgenic **A**denocarcinoma of the **M**ouse **P**rostate) mice as our model. The control group of mice were fed with a western diet to mimic the common high-fat diet eaten by U.S. men. Three additional groups of mice were fed the western diet supplemented with 0.04%, 0.2% and 1% DIM in diet. Incidence of advanced PCa [poorly differentiated carcinoma (PDC)] in the control group was 60%. 1% DIM dramatically reduced PDC incidence to 24% ($p=0.001$), while 0.2% and 0.04% DIM reduced PDC incidence to 38% ($p=0.056$) and 45% ($p=0.18$) respectively. However, DIM did affect mice weight, so to eliminate any possible confounding effect of body weight on PDC incidence, we did logistic regression with our data. This showed a clear negative association between DIM concentration and PDC incidence with $p=0.004$, while the association between body weight and PDC incidence is not significant ($p=0.998$). Offsetting this confirmation of our hypothesis, there was also a trend of increasing well differentiated carcinoma (WDC) along with the increase of DIM concentration in the diet. 1% DIM increased WDC incidence from 40% to 66% ($p=0.02$)

when compared to control, while other diets had no statistically significant effects. In conclusion, our results show that dietary DIM can inhibit the most aggressive stage of prostate cancer.

Introduction

Prostate Cancer is the most prevalent cancer among men in the United States and about one man in nine will get prostate cancer during his life time. It is estimated that about 164,690 new cases of prostate cancer will be diagnosed in 2018 and that more than 29,000 patients will die from this disease, making it the second leading cause of death in men [165]. Though there is high incidence for prostate cancer, the majority (91%) of them will be diagnosed as localized cancer. The 5-year relative survival rate in a patient with local cancer is nearly 100%, while that in patients with metastatic prostate cancer is only 30% [166]. Our goal in this study was to study DIM's effect in preventing advanced prostate cancer, which is PDC in the TRAMP mouse model.

DIM has been tested in 16 clinical trials for treating different types of diseases, including prostate cancer, breast cancer, thyroid disease, cervical Dysplasia, and others [92, 93, 103, 167-178]. These studies have suggested the safety and potential of DIM being used as a drug candidate for treatment of disease. More clinical trials with large sample size and longer periods are needed to confirm the effect of DIM. Meanwhile, study about the

mechanism of how DIM works is also very important. Previous studies have shown that DIM can inhibit cancer through several pathways, such as the estrogen receptor signaling pathway, aryl hydrocarbon receptor pathway, androgen receptor signaling pathway, Nrf2 pathway and NF-κB pathway, suggesting DIM a multi-target drug [79, 81, 98, 100, 107, 109, 121]. Our lab previously showed that in TRAMP mouse model, knocking out ERβ doubled the incidence of advanced prostate cancer (PDC), while knocking out ERα decreased PDC incidence almost to zero [43]. Thus, ERα is promoting PDC and ERβ is preventing PDC. We have shown in Chapter II that DIM stimulates estrogen receptor activity. Based on this, we **hypothesized** that DIM would prevent PDC in TRAMP mouse model though modifying estrogen receptor activity.

To test this hypothesis *in vivo*, two studies are needed. First, examine DIM's effect on preventing PDC in TRAMP mice. Second, investigate the PDC-preventing effect of DIM in double transgenic ER knockout/TRAMP mice. In this chapter, we only talk about the results from this first study (Study two will be discussed in chapter IV).

Several *in vivo* animal studies previously showed DIM could inhibit prostate cancer in TRAMP mice [109, 179-181]. Nachshon-Kedmi, M. et al. and Fares, F. et al. used a xenograft model by subcutaneous injecting a mouse prostate cancer cell line TRAMP-C2 into C57BL/6 mice [180, 181]. However, this model might not be appropriate to assess

the preventive effect of DIM on advanced prostate cancer development. Cho, H.J. et al examined the effect of DIM on prostate cancer development by feeding DIM to TRAMP mice [179]. In their report, DIM decreased well differentiated carcinoma (WDC) while increasing the incidence of prostatic intraepithelial neoplasia (PIN). Incidence of PDC was not examined. Since we were interested in DIM's effect on advanced prostate cancer, which is PDC in TRAMP mice, and PDC can arise independently, not following the progression from WDC to PDC, an additional study assessing DIM's effect on PDC incidence in the TRAMP model is necessary. Wu, T.Y. et al showed that 1% DIM in the diet could decrease the incidence of PDC in TRAMP mice [109]. However, only one concentration (1%) was used in their study. As we know, hormesis is common in hormone receptors responses, and it presents with an Inverted-U shaped dose- response curve. With only one concentration in the previous studies, we could not decide which part of the response curve this concentration (1% DIM) represented [182-184]. Investigating the ability of DIM to prevent PDC under various concentration is important for determination of concentrations used for the double transgenic ER knockout/TRAMP mice study as well as other future studies.

We decided to use a high fat diet to mimic the western diet eaten by American males. The physiological concentration of DIM in plasma ranged from a high of 16uM to a low of ~ 0.3uM over 24 hours, following a one-time gavage consumption of 250mg/kg in mice

[185]. In their case, a 30g mice would consumed 7.5mg DIM for 24h. We decided to use 1% DIM as the highest concentration, which should give mice a daily DIM consumption around 30mg, 4 times over the reported gavage study. Two additional lower doses, 0.2% and 0.04% were added to our study (Table III-2). These three dosages should fit in the concentration range we tested in Chapter II, showing DIM could inhibit TRAMP-C2 cell growth between 10-90uM. We hypothesized that DIM would prevent PDC in TRAMP mice model dose-dependently.

Materials and Methods

TRAMP mice

Animal study protocols were approved by the Animal Care and Use Committee of the University of Missouri and all institutional guidelines for animal welfare and use were followed. Study mice were bred by crossing male FVB TRAMP mice with C57BL/6 female mice to generate F1 C57/FVB hybrid male TRAMP mice as described previously [186]. TRAMP positive F1 males received study diets starting from 6 weeks of age, as TRAMP mice begin forming tumors after the onset of puberty (usually around 8 weeks of age) and continued until study termination. Study mice were housed 2-3 per micro-isolator cages and given free access to food and water, under the condition of a daily light:dark cycle of 12:12h, ambient temperature at 21℃, and humidity at 50% throughout the study. Mice were weighed every week to monitor body weight and tumor

burden. At the age of 20 weeks, mice were euthanized, and their tissues were weighed and collected for further analyses. Prostates were fixed in 10% neutral buffered formalin and then embedded with paraffin. Tissue sections were stained with hematoxylin and eosin and were scanned by veterinary pathologists to determine cancer stages in a blinded fashion as previously described [186] . Prostates were staged as one of the following designations: 1) normal, 2) hyperplasia, 3) prostatic intraepithelial neoplasia (PIN), 4) well differentiated carcinoma (WDC), 5) moderately differentiated carcinoma (MDC) and 6) poorly differentiated carcinoma (PDC). Based on the findings by Bailman, et. al., PDC can arise independently from hyperplastic epithelial lesions and represents the primary malignant cells in the TRAMP model [187]. Because of these findings, if a small portion of the prostate had a poorly differentiated morphology, it was scored as PDC.

Diet Formulations

A modified high fat, low cholesterol diet based on the formulation of Western Diet (D12079B) from Research Diets, Inc (New Brunswick, NJ) was used, by removing the added 0.15% cholesterol (table III-1). The fat content in the diet was 21% w/w. DIM in the diet was purchased from BulkSupplements.com (Bulk-DIM) (Henderson, NV) and its purity was verified by HPLC. Using DIM purchased from Sigma Aldrich (Sigma-DIM) (St. Louis, MO) as a standard, whose purity >98%, Bulk-DIM showed 100% of the standard (Figure III-1). In addition, we also tested the ability of Bulk-DIM to stimulate

estrogen receptor transcriptional activity, which showed no difference with the Sigma-DIM (Figure III-2). There was a total of four groups of mice on study with 39-42 mice per treatment group. Except for the control, three doses of DIM, 0.04%, 0.2% and 1% w/w, were mixed and pelleted into the high fat diet by Research Diets, Inc (New Brunswick, NJ) as well. All diets were stored at -20℃ to prevent oxidation and spoilage.

ER Knockout (KO) mice

To generate the transgenic ERα/βKO mice, we crossed female C57Bl/6J (or FVB) mice, heterozygous for the ERα or ERβ gene with male C57Bl/6J (or FVB) mice that were heterozygous for the ERα or ERβ gene as previously described [43]. ERα/β wild type (WT), ERα/β heterozygous (Hetero) and ERα/βKO offspring were used in this study. Study mice were housed 2-3 per micro-isolator cages and given free access to food and water, under the condition of a daily light:dark cycle of 12:12h, ambient temperature at 21℃, and humidity at 50% throughout the study.

Weight study in transgenic ERKO mice

ERWT, ERα/β heterozygous or ERα/βKO mice with similar age, same background, and same gender were set as genopairs and each genopair was housed in one cage. Since we used C57/FVB hybrid TRAMP mice in previous study. ERKO mice in both C57Bl/6J and FVB background were used. The ages of study mice ranged from 6 weeks to 17 weeks,

so that if in any case, the weight loss caused by DIM was only effective at younger age, we would be able to notice that. (Table III-3)

1. **Study 1- Feeding**

Mice with different ER genotypes were divided into two groups, fed with either the high fat control diet or 1% DIM from previous study. Mice body weights were measured daily to monitor the weight change.

2. **Study 2- Injection**

We used the same mice from study 1. After study 1 finished at day 10, all mice were moved to the control diet to let mice recover the weight gain for 39 days. Study 2 stared at day 50 and lasted for two weeks. DIM dissolved in corn oil at 40mg/ml. Mice were intraperitoneal injected three times per week with either corn oil or 5ul/g mouse body weight DIM solution, making the final concentration to be 200mg/kg mouse body weight. DIM solution was made fresh each time and mice body weights were measured every time before injection.

Measurement of food consumption

Study diets were provided as pellets and placed in a jar that allowed mice to climb into to get the food. A wire entanglement was placed above the food to prevent mouse taking the food pellets out of the jar. The food jar was measured every day to calculate the daily food consumption per mouse.

Results

DIM prevented advanced prostate cancer development in TRAMP mouse

Study mice were placed on a high fat diet to mimic the western style high fat diet among U.S males. The three experiment groups were supplemented with 1%, 0.2% and 0.04% (w/w) DIM. Incidence of PDC, which is the most aggressive stage of prostate cancer in TRAMP mouse, in the control group was 60%. By adding DIM to the diet, we saw a clear trend line of decreasing PDC incidence, with 0.04%, 0.2% and 1% DIM reducing PDC incidence to 45% (P=0.14), 38% (P=0.047) and 24% (p=0.0012), respectively (Table III-4). To our surprise, we also observed an increase in Well Differentiated Carcinoma (WDC), though this increase was only significant in mice treated with 1% DIM (p= 0.028). The overall incidence of cancer, which included WDC, MDC and PDC, did not change between groups. 1% DIM had a slightly lower cancer incidence of 91% and it was not statistical significant from other groups. (Table III-5).

DIM caused weight loss in TRAMP mice fed with high fat diet

We observed an effect of DIM on mouse body weight in mice fed with 1% DIM (Figure III-3). At age 20 weeks, mice in the 1% DIM group had a lower average body weight of 29.5g compared to the control (34.9g) (Figure III-3B). During the weekly monitoring of weight, we found that mice treated with 1% DIM had a dramatic decrease in body weight,

after the first week of treatment. Though they seemed to recover at the second week, they were never able to catch up with the other groups (Figure III-3C). Epidemiological studies have suggested that obesity positively associates with the risk of aggressive prostate cancer [188, 189]. In order to eliminate any possible confounding effect of body weight on PDC incidence, we fit our data to a logistic regression using both body weight and dosage of DIM as the independent variables and PDC incidence as the dependent variable. The result of the logistic regression indicated that the incidence of PDC decreased as we increased the dose of DIM, even controlling for body weight. The association between PDC incidence and DIM dosage was statistically significant ($p=0.004$), while the association between PDC incidence and body weight was not significant ($p=0.953$) (Figure III-4).

The difference in body weight seen between mice in 1% DIM and other groups can partly be attributed to the differences between the prostate and testes fat pad. There were no statistical differences seen for brain, liver and kidneys among groups (Figure III-5A, B and E). All groups had very similar mean size of testes, though 0.2% DIM had a slightly bigger average weight compared to the control and 1% DIM (Figure III-5D). 1% DIM mice had smaller fat pads than mice in other groups (Figure III-5C). Treatment of DIM decreased the number of big prostates over 1g as well as increased the number of small prostates below 0.1g (Figure III-5F). There was a trend of decreasing mean prostate size

when we increased the concentration of DIM, with 2.5g in control, 0.97g in 0.04% DIM, 0.78g in 0.2% DIM and 0.52g in 1% DIM group. However, only control mice had a statistical significant larger mean prostate size compared to other groups. No statistical differences were found among the three DIM treatment groups when compared to each other.

Weight loss was caused by mice disliking the taste of DIM

We tested DIM in transgenic ERKO mice to determine the role of estrogen receptors in mediating weight loss caused by DIM. In Study 1- Feeding, all mice fed with high fat control diet had relatively stable weight curve, either keeping flat or increasing a little bit, depending on the age of the mice. A dramatic drop of body weight was observed in mice fed with 1% DIM in the first 1-4 days no matter what genotypes the mice were (Figure III-6). Therefore, the weight loss was caused by only DIM but not through any estrogen receptor mediating effects.

Dramatic weight loss happened in the very early days when mice were given DIM diet, and mice seemed to get used to this “DIM effect”, with recovered and stabilized body weights after that (Figure III-6). Also, because of the bad smell of DIM, which smells like mothballs, we hypothesized that the weight loss was caused by mice disfavoring the taste of DIM. In study 2- Injection, ERKO mice genopairs were injected with either 200mg/kg

DIM or corn oil as control three times a week for two weeks. Daily weight monitoring indicated all mice had very similar weight curve, independent of the treatment (Figure III-7). Thus, DIM caused weight loss was only observed when DIM was orally consumed by mice.

We then compared the average daily food intake between mice in high fat control and 1% DIM diet groups during the first two weeks on study. 1% DIM mice had an average food consumption around 2g per mouse per day, while mice in control group had about 3.6g per mouse per day (Figure III-8). Taken together, this suggested that the weight loss in 1% DIM group was because mice didn't like the taste of DIM.

Discussion

For years, prostate cancer keeps being on the top of the cancer prevalence and death list for men. Besides the well-known risks such as aging and genetic reason, diet can also impact the development of prostate cancer. Epidemiological data has demonstrated that men in the U.S. and other developed countries, who usually have a western style high fat diet, have a much higher risk for prostate cancer [127]. Japanese and Chinese men have an increased risk for prostate cancer, after they emigrate and adopt a more western diet [190, 191]. In this study, a high fat diet was used to best mimic and test the effects of DIM on advanced prostate cancer development among American men as a dietary

supplement.

We were very excited to see that DIM inhibited PDC dose dependently. The increase in WDC incidence was surprising initially. However, this increase is not real, but instead is just the bias of our method for scoring the cancer stage. PDC arises independently of epithelial changes, and in our experience, small foci of PDC expand to involve all lobes of the prostate given enough time. Using the standard staging methodology, identification of PDC in any prostate lobe, regardless of the status of epithelial hyperplasia or neoplasia in the dorsolateral prostate lobe, is scored as PDC. Thus, a prostate with a mixture of a large portion of WDC but a small focus of PDC is determined to be PDC. Based on this approach, it is possible that the overall incidence of WDC is not changed, but rather that PDC incidence is decreasing. There is no statistical difference in the overall incidence of cancer (WDC, MDC and PDC combined together) between all treatment groups. Thus DIM decreased the incidence of PDC, which might reveal the WDC that used to be "covered" by PDC, and there just seemed to be an increase in WDC incidence. A possible future solution to decrease WDC would be to combine DIM and another compound that inhibits WDC in the diet, for example, genistein, which we showed previously only inhibited WDC and had no effect on PDC [43].

Four previous studies showed that DIM could inhibit prostate cancer in TRAMP model

[109, 179-181]. However, because of the lack of examination on PDC in some studies as well as the hormesis effect, we decided to go with our own DIM study on TRAMP mice. Interestingly, there is another report using DIM in C57/FVB F1 hybrid TRAMP mouse as we did and showed very minimal effects of DIM in those mice [192]. In the study, mice were gavage fed with the highest of 100mg/kg DIM three times per week, which was <3mg for 48hrs for a 30g mice. Compared to our study that mice would consume around 30mg per 24hrs, it was more than 20 times lower than our highest concentration. Thus, the highest concentration of DIM they used in the study was about the same as our 0.04% DIM in diet, at which we did not see a statistically significant effect of DIM either. Besides that, the way we measured the prostate cancer protection efficacy was different. In their study, they sacrificed mice when a palpable tumor was detected in the prostate cancer, thus to compare the palpable prostate tumor-free survival, and only measured the incidence of neuroendocrine prostate carcinomas (NEPC) and metastasis from that [192]. Two variables in their study, including the different concentration of DIM and various length of treatment time made it complicated to compare the efficacy of DIM among groups. While for other studies including ours, all mice received or did not receive DIM treatment for the same length of time and incidence of cancer in different stages were compared among groups [109, 179-181].

Previous studies of DIM in TRAMP mice did not report the influence of DIM on body

weight [109, 179-181]. There are two reasons that may explain the difference between our results and these previous reports. First, they used low fat diets which contained no more than 11% w/w fat in the diet. In contrast, we used a high fat diet that consisted of 21% w/w fat. DIM is reported to suppress obesity when fed with a high fat diet [193]. Second, the genetic background of our study mice was different than others. We used the C57/FVB F1 hybrid TRAMP mouse, while most of them used C57BL/6 TRAMP mice.

In chapter II, we demonstrated that DIM stimulated estrogen receptors. Because ERβ is reported to alleviate obesity [194], we wanted to know whether the initially effect of DIM on body weight was mediated by ERβ. We treated mice with DIM through two different methods: feeding mice with diet containing 1% DIM or IP injecting to let DIM absorb to the bloodstream directly. If the initial weight loss by DIM was mediated through ERβ, we should observe weight loss in DIM-treated WT mice, while no weight loss with ERβKO mice. DIM treated ERβHetero mice might have a moderate weight loss compared to the WT mice. However, we observed no weight change difference among mice with different ER status, suggesting ER was not involved in the initial weighted loss caused by DIM during the first few days on study. Moreover, comparing results from Study 1- Feeding with Study 2- Injection, we found that the body weight change was only observed when DIM was orally administrated to mice. Unlike I3C, which would condense in the stomach acid and form DIM, DIM was very stable during digestive process, so the weight loss

was unlikely caused by the metabolism of DIM. Thus, we **hypothesized** that the initial weight loss was caused by decreased food consumption. We measured the average daily food intake per mice in both 1% DIM and control groups during the first two weeks on study. The average daily food consumption in the 1% DIM group was much lower than that of control (Figure III-7). Altogether, this suggests that the initial drop in body weight of mice in the 1% DIM group is not related to the estrogen receptor, but likely because of the taste of the diet which lowers the food intake.

Results from ERKO mice Study 1- Feeding and Study 2- Injection explained the weight loss, which occurred at the beginning of the study. Since mice did not like the taste of DIM, they tried to eat as little as they could only to meet the minimal living requirement. However, this might not be the only reason that resulted the weight difference between mice in 1% DIM group and other groups at the end of our study. Yang, H., et al reported that gavage fed mice with 50mg/kg body weight DIM in high fat diet for 12 weeks would suppress the obesity through inhibiting adipogenesis [193]. DIM itself would cause the weight decrease in mice. Therefore, the weight difference between 1% DIM group and others could be a combination of both decreased food intake and effects of DIM on adipose tissue.

For future DIM mice studies, we should avoid the decrease of initial food intake caused

by DIM's taste. There are several possible solutions. Instead of adding DIM to the diet pellet, we could gavage feed the mice. Since we usually have very large groups of mice on study, this will be very time-consuming. Another way will be using lower concentrations of DIM and adding sugar to the diet to make mice like the teste of the diet.

In conclusion, we demonstrated that DIM dose-dependently prevented advanced prostate cancer development when fed high fat diets. Future studies to confirm ER's importance in DIM's function, including double transgenic ER knockout/TRAMP mice studies will need to be performed.

Formula of high fat diet		
%	**gm**	**kcal**
Protein	20	17
Carbohydrate	50	43
Fat	21	41
Total		100
kcal/gm	4.7	
Ingredient	**gm**	**kcal**
Casein, 80 Mesh	195	780
DL-Methionine	3	12
Corn Starch	50	200
Maltodextrin	100	400
Sucrose	341	1364
Cellulose, BW200	50	0
Milk Fat, Anhydrous	200	1800
Corn Oil	10	90
Mineral Mix S10001	35	0
Calcium Carbonate	4	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
Ethoxyquin	0.04	0
Total	1001.54	4686

Table III- 1: Formulation of high fat diet

Group	control	0.04% DIM	0.2% DIM	1% DIM
Estimated DIM consumption for 24h	0	1.2mg	6mg	30mg
Number of mice per group	42	42	39	41

Table III-2: Estimated 24h DIM consumption in all groups.
Assume that mice had an average food intake of 3g per day.

Design for weight study in ERKO mice						
Time period (days)						
1-10 (study 1-Diet)	11-49	**50-64 (Study 2-Injection)**	**Genotype**	**Age (weeks)**	**Gender**	**Background**
1% DIM diet	Control diet	**DIM in corn oil (200mg/kg)**	ERβKO	16	M	C57Bl/6J
			ERβWT	14		
			ERβKO	8	F	
			ERβWT	8		
			ERβHetero	8	M	
			ERβKO	8		
			ERβHetero	6		
			ERaKO	17	F	
			ERaWT	17		
			ERaKO	6	F	
			ERaHetero	6		
			ERaWT	6		
			ERaKO	6	F	
			ERaWT	6		
			ERβWT	10	M	FVB
			ERβHetero	10		
			ERβKO	10		
Control diet		**Corn oil**	ERβKO	13	M	FVB
			ERβWT	13		
			ERaHetero	6	F	C57Bl/6J
			ERaHetero	6	M	
			ERaKO	6		
			ERβWT	8	M	
			ERβHetero	8		
			ERβWT	8		
			ERβHetero	6	M	

Table III- 3: Design for weight study in double transgenic ERKO TRAMP mice

Effects of DIM on the incidence of prostate cancer in 20-week-old TRAMP mice

Group	N	Tumor Stage					
		Non-cancer n (%)			Cancer n (%)		
		Normal	Hyperplasia	PIN	WDC	MDC	PDC
Control	42	0	0	0	17 (40%)	0	25 (60%)
0.04% DIM	42	0	0	0	23 (55%)	0	19 (45%)
0.2% DIM	39	1 (3%)	0	0	23 (59%)	0	15 (38%) [a]
1% DIM	41	0	1 (2%)	3 (7%)	27 (66%) [c]	0	10 (24%) [b]

Table III- 4: DIM inhibited PDC incidence in a dose-dependent manner.

The inhibitory effect of 0.04% DIM was not significant (p=0.14). Both 0.2% DIM (p= 0.047)[a] and 1% DIM (p=0.0012)[b] had a statistically significant effect on PDC, when compared to the control. There was also a trend line of increasing WDC incidence, but only 1% DIM had a statistical effect (p=0.028)[c]. Incidence of tumor stage were analyzed using Fisher's exact one-tailed *P* value

DIM's effect on overall cancer incidence

Diet	N	Non-Cancer	Cancer
Control	42	0	42 (100%)
0.04% DIM	42	0	42 (100%)
0.2% DIM	39	1(3%)	38(97%)
1% DIM	41	4(9%)	37 (91%)

Table III- 5: Overall cancer incidence in DIM TRAMP mice study.

Non-cancer = sum of normal, HYP, and PIN; Cancer = sum of WDC, MDC, and PDC. Mice in 1% DIM group had a slightly lower cancer incidence, but it was not statistical significant (p=0.055).

Figure III- 1: HPLC verification of the purity of DIM from BulkSupplements.com

DIM Test No. 2

D-7000 HPLC System Manager Report

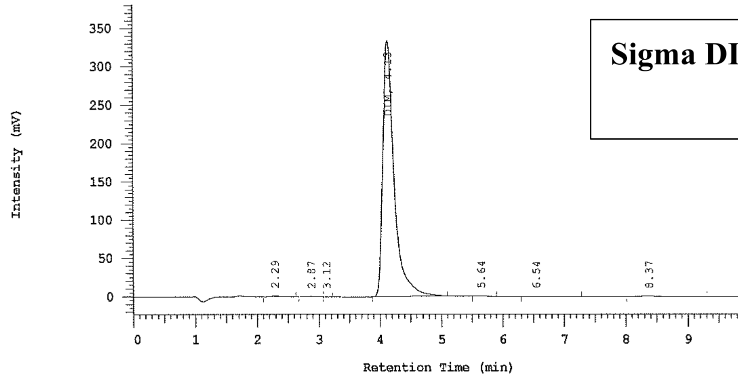
Analyzed: 06/16/15 12:24 PM

Reported: 06/17/15 09:49 AM
Processed: 06/17/15 09:49 AM

Data Path: C:\WIN32APP\HSM\samples2014\DATA\1398\
Processing Method: DIM

System(acquisition): Sys 1
Application: samples2014
Sample Name: STD DIM 25ppm
Injection from this vial: 1 of 1
Sample Description:

Series:1398
Vial Number: 1
Vial Type: STD2
Volume: 20.0 ul



Sigma DIM – Standard 1

Name	RT	Area	Conc 1 ppm	Conc 2 ppm
DIM	4.13	4255710	25.0000	25.0000
		4255710	25.0000	25.0000

Peak rejection level: 10

Peak Quantitation: AREA

Page Indicator 1 / 5

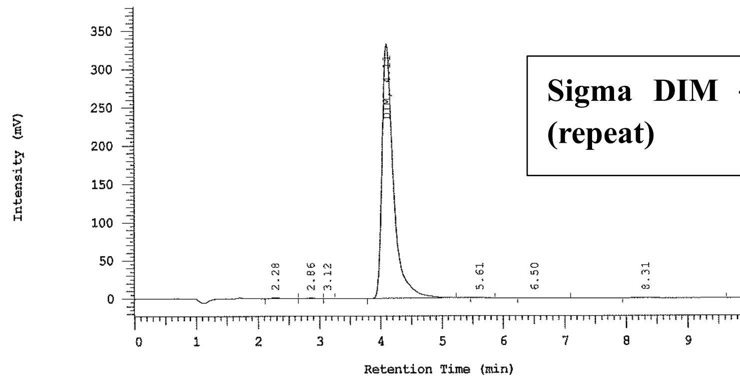
D-7000 HPLC System Manager Report

Analyzed: 06/16/15 12:57 PM
Reported: 06/17/15 09:49 AM
Processed: 06/17/15 09:49 AM

Data Path: C:\WIN32APP\HSM\samples2014\DATA\1398\
Processing Method: DIM

System(acquisition): Sys 1
Application: samples2014
Sample Name: STD DIM 25ppm
Injection from this vial: 1 of 1
Sample Description:

Series:1398
Vial Number: 1
Vial Type: STD2
Volume: 20.0 ul



Sigma DIM – Standard 2 (repeat)

Name	RT	Area	Conc 1 ppm	Conc 2 ppm
DIM	4.11	4274957	25.0000	25.0000
		4274957	25.0000	25.0000

Peak rejection level: 10

Peak Quantitation: AREA

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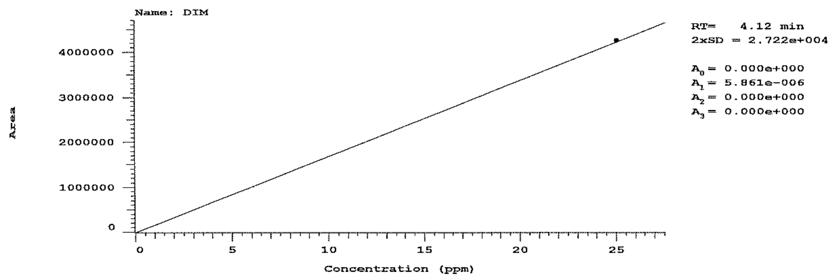
Calculation Method: EXT-STD (Bracketing Mode)

Peak Quantitation: AREA

Name	Pts	A0	A1	A2	A3	2 x SD	Units
DIM	2	0.000E+00	5.861E-06	0.000E+00	0.000E+00	2.722E+04	ppm

Least Squares Analysis
Order: Linear - f(Response)
Through zero: YES
Weight factor: 1.0

Component Name	RT (min)	Window (%)	STD1 (ppm)	STD2 (ppm)
DIM	4.13	10.00	100.000	25.0000



Sigma DIM standard curve

D-7000 HPLC System Manager Report

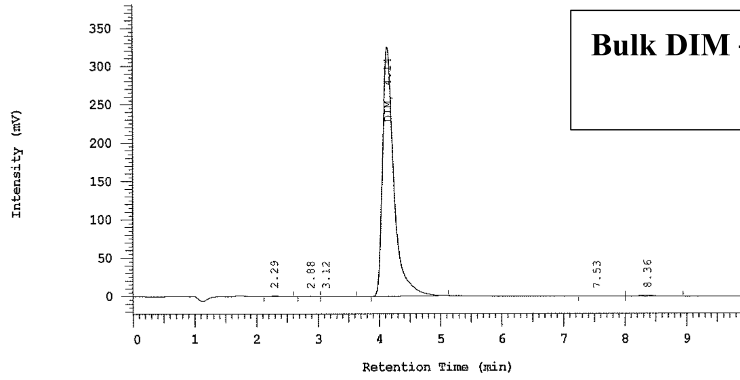
Analyzed: 06/16/15 12:35 PM

Reported: 06/17/15 09:49 AM
Processed: 06/17/15 09:49 AM

Data Path: C:\WIN32APP\HSM\samples2014\DATA\1398\
Processing Method: DIM

System(acquisition): Sys 1
Application: samples2014
Sample Name: BULK 1 OLD 25ppm
Injection from this vial: 1 of 1
Sample Description:

Series:1398
Vial Number: 2
Vial Type: UNK
Volume: 20.0 ul



Bulk DIM – Test 1

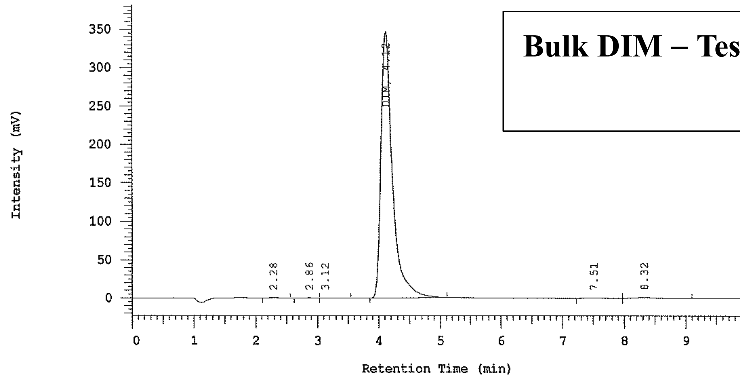
Name	RT	Area	Conc 1 ppm	Conc 2 ppm
DIM	4.14	4162387	24.3965	24.3965
		4162387	24.3965	24.3965

Peak rejection level: 10

Peak Quantitation: AREA

D-7000 HPLC System Manager Report

Analyzed: 06/16/15 12:46 PM
Reported: 06/17/15 09:49 AM
Processed: 06/17/15 09:49 AM
Data Path: C:\WIN32APP\HSM\samples2014\DATA\1398\
Processing Method: DIM
System(acquisition): Sys 1
Series:1398
Application: samples2014
Vial Number: 3
Sample Name: BULK 2 NEW 25ppm
Vial Type: UNK
Injection from this vial: 1 of 1
Volume: 20.0 ul
Sample Description:



Bulk DIM – Test 2 (repeat)

Name	RT	Area	Conc 1 ppm	Conc 2 ppm
DIM	4.12	4460508	26.1438	26.1438
		4460508	26.1438	26.1438

Peak rejection level: 10

Peak Quantitation: AREA

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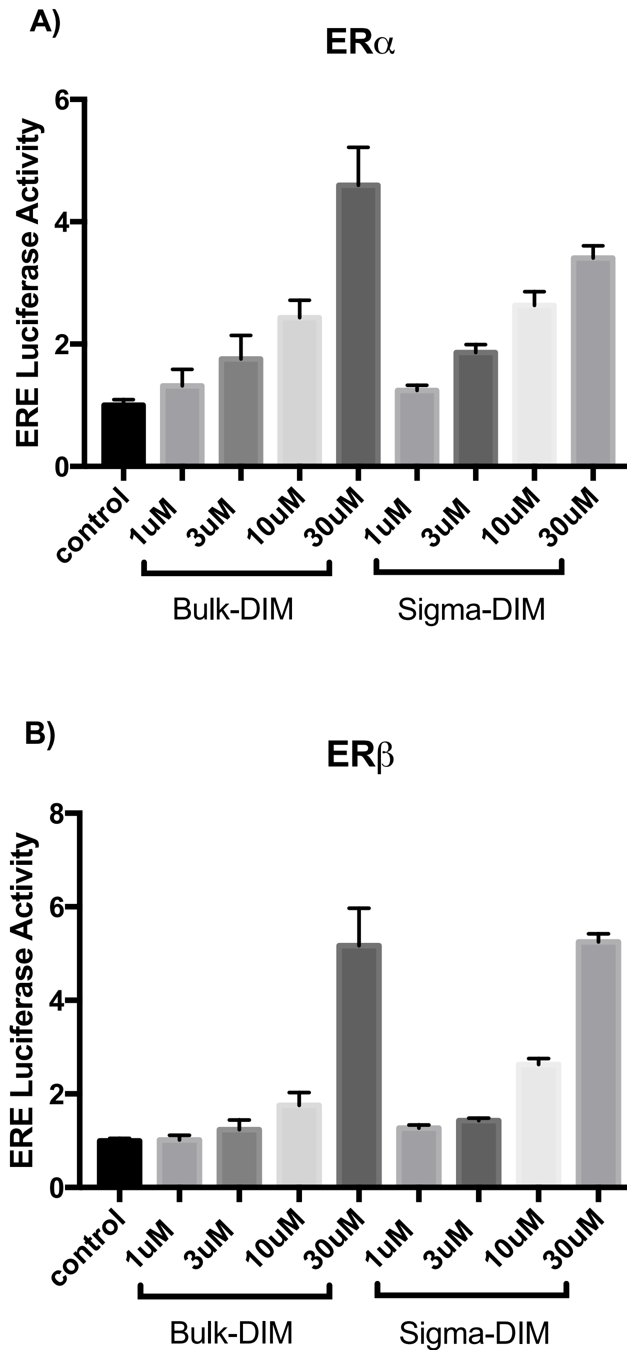

Figure III- 2: Comparison of Bulk-DIM and Sigma-DIM estrogenic activity

Bulk-DIM and Sigma-DIM were tested parallel for their abilities to activate estrogen receptor transcriptional activity in a ERE luciferase assay in U2OS cells. Error bars indicates S.E.M.

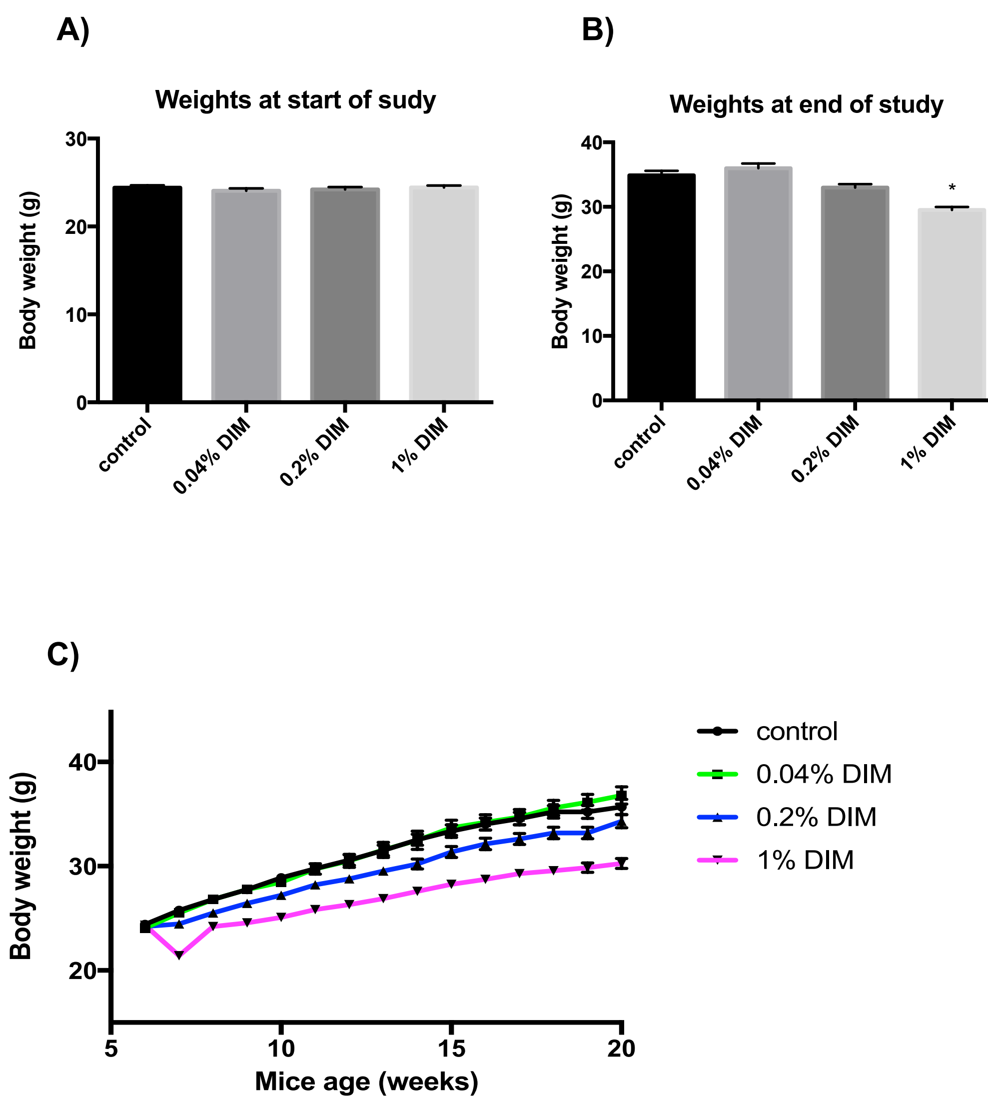

Figure III- 3: Effect of DIM on average mouse body weight.

A) Average body weight of each group at the beginning of the study. **B)** Average body weight of each group at the end of the study. **C)** Time course change of the body weight. All mice on study were measured weekly and average body weight was calculated for each group. Error bars indicates S.E.M. * indicates p< 0.01

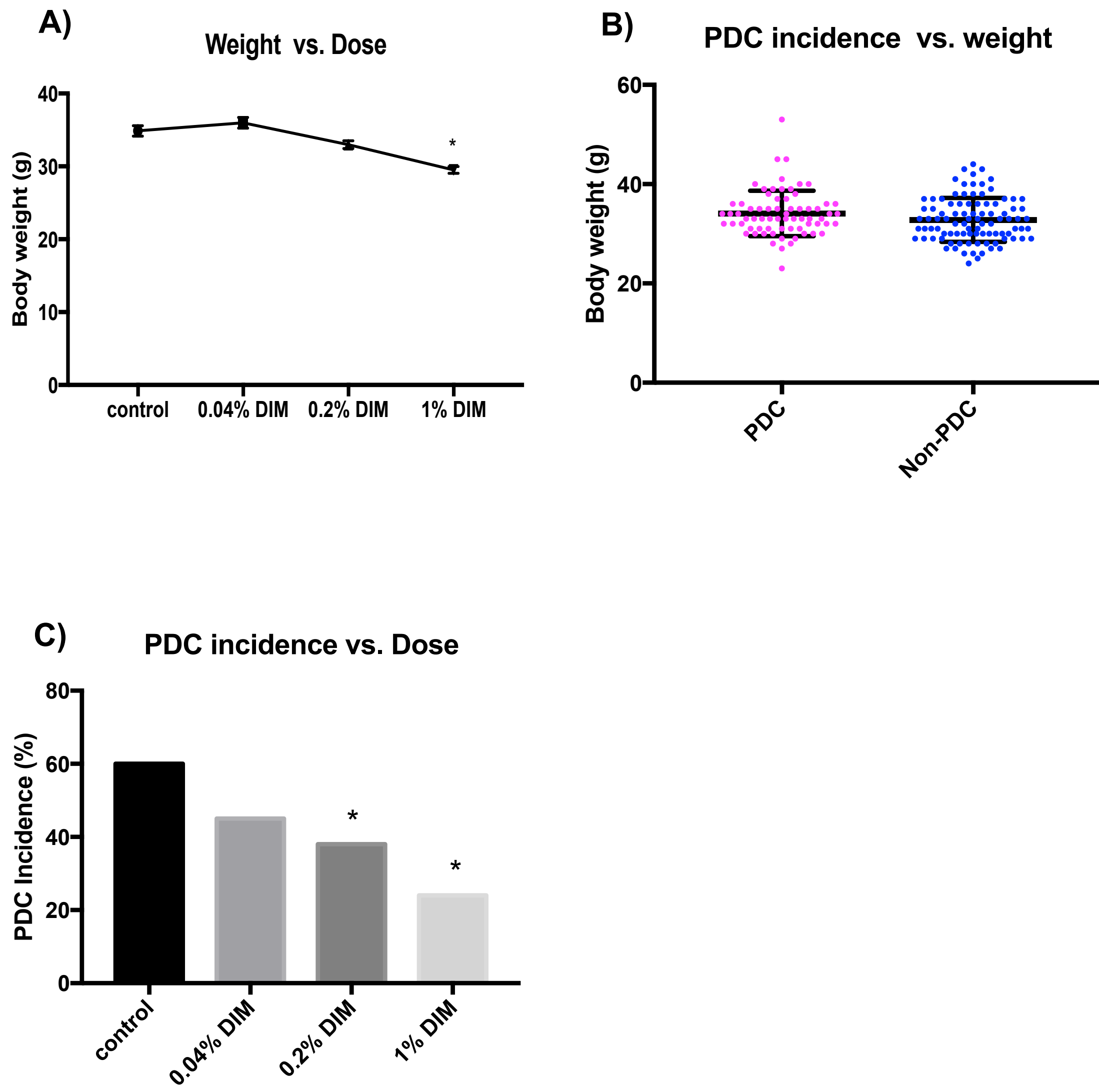

Figure III- 4: Correlation between mouse body weight, dosage of DIM in the diet and PDC incidence.

Mice body weights were measured at the end of study (age of 20 weeks). **A)** Average mouse body weight decreased as increasing DIM concentration in the diet. Error bars indicates S.E.M. **B)** Average body weights of mice with PDC vs. without PDC. Error bars indicates S.D. **C)** Increasing DIM concentration in the diet decreased PDC incidence. Error bars indicates S.E.M. * indicates $p < 0.01$

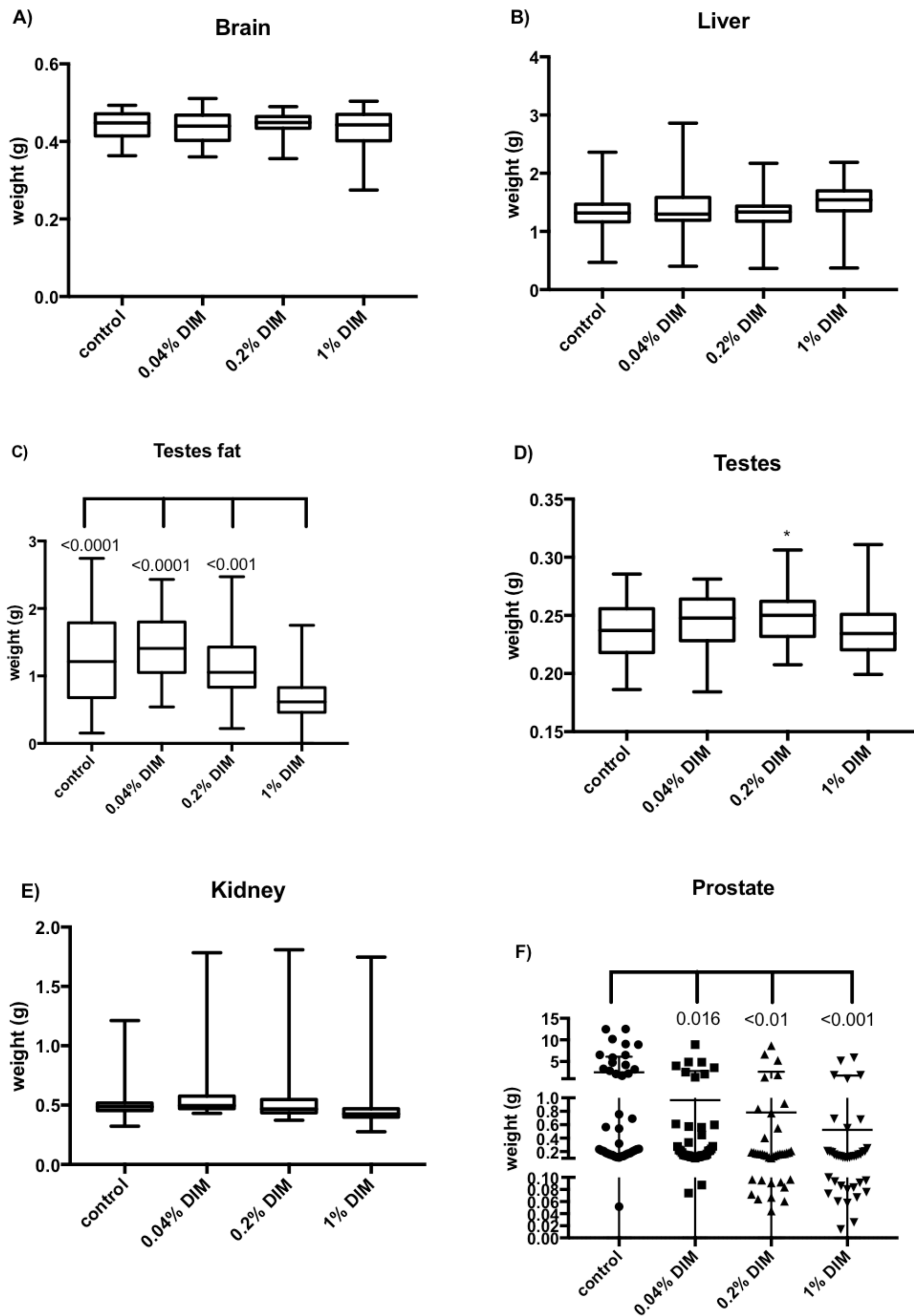

Figure III- 5: Comparisons of weights of different organs in DIM TRAMP mice study.

A) Brain, **B)** Liver, **C)** testes fat pad, **D)** testes, **E)** prostate. Whiskers represent the min. and max. values. * indicates $P<0.05$. **F)** weight distribution of prostate in each group.

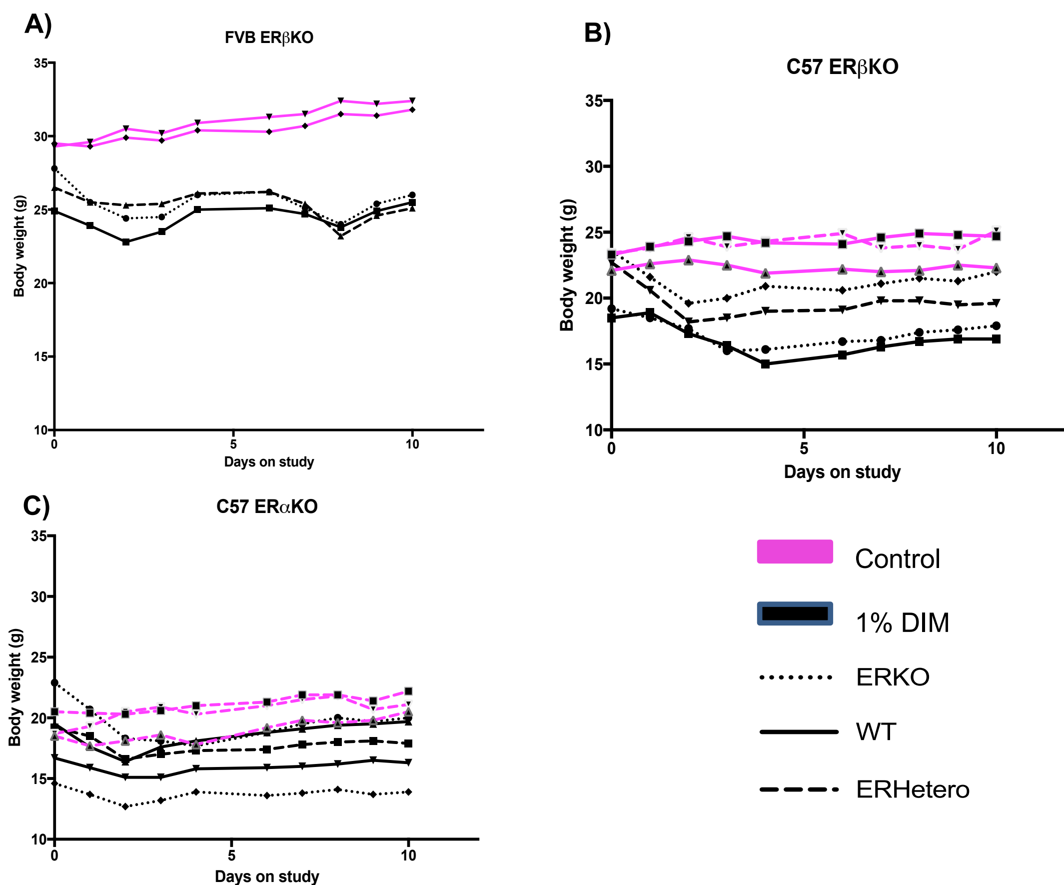

Figure III- 6: Time course body weight change of ERKO mice study 1-feeding

Mice were fed with either high fat control or 1% DIM diet. Mouse body weight was measured every day for 10 days. Each line represents one mouse. Solid line (—) is wild type mouse; dashed line (--) is ER hetero genotype mouse and dotted line (······) is ER knockout mouse. Mouse fed with control diet is in pink color and mouse fed with 1% DIM diet is in black color.

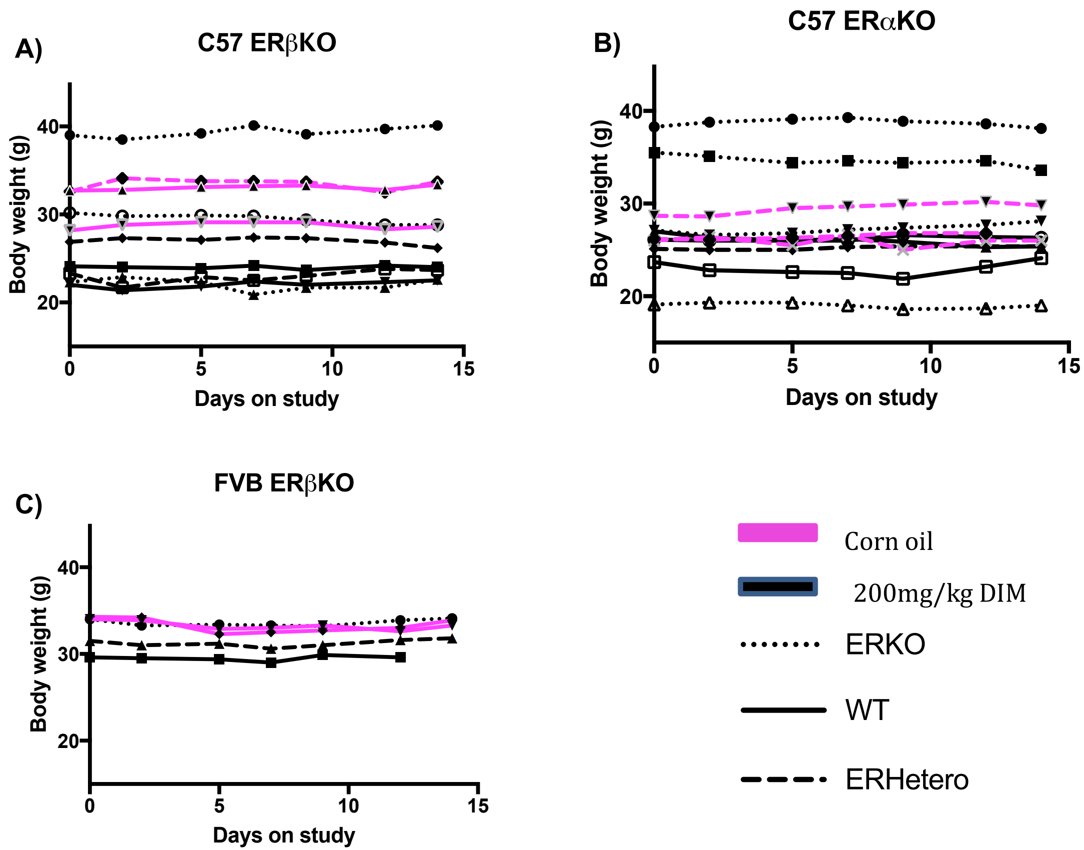

Figure III- 7: Time course body weight change of ERKO mice in study 2-injection.

Mice were Intraperitoneal injected with corn oil or 200mg/kg DIM. Mouse body weight was measured every day for 15days. Each line represents one mouse. Solid line (—) is wild type mouse; dashed line (--) is ER hetero genotype mouse and dotted line (┈) is ER knockout mouse. Mouse injected with corn oil is in pink color and mouse injected with 1% DIM diet is in black color.

Figure III- 8: Comparison of average food intake between mice in control and 1% DIM group.

Food consumption of control (N=9) and 1% DIM (N=17) treated mice in the ERKO feeding study were measured daily over the first two-week period. Error bars indicates, S.E.M. * indicates $p < 0.01$.

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CHAPTER IV

SUMMARY AND PERSPECTIVES ON FUTURE RESEARCH

DIM has been widely used as a dietary supplement in recent years and has been shown to have great therapeutic potentials for many diseases, such as prostate cancer, breast cancer, thyroid disease, cervical dysplasia, and others [167-171]. Many publications show that DIM interacts with many pathways, including the estrogen receptor-signaling pathway [79, 81, 98, 100, 107, 109, 121]. However, the primary mechanism of how DIM works to inhibit cancer and the role of estrogen receptor during this inhibition is still not clear.

In Chapter II of this dissertation, we reported the ligand independent activation of both ERα and ERβ by DIM. This is contradictory to the report by Vivar, O. I. that DIM selectively activated ERβ [98]. Though we tried hard to eliminate any possible experimental differences between us and them, we could not repeat DIM's selectivity for ERβ as described in their paper. Moreover, The authors could not provide us the ER expression plasmids or cell lines used in the paper when we contacted them. It is still a mystery and a pity that we did not figure out what was present in their experiments to

inhibit the stimulation of ERα by DIM. Since we both used cell lines with no ER expression and measured ER transcriptional activity after either transient or stable transfection of ER into the cells. In order to confirm DIM's stimulation of endogenous ER activity, quantitative real time PCR (qRT-PCR) is required to be done with cell lines expressing ER after the treatment with DIM, especially in the prostate cancer cell lines, like human prostate cancer PC3 cells and mouse prostate cancer TRAMP-C2 cells.

We found out that DIM unexpectedly stimulated both ERs, however, when we measured DIM's binding to ERβ in a ^{3}H-E2 competitive binding assay, we did confirm that DIM did not compete with E2. This is surprising and it will be interesting to figure out how DIM stimulate ER without "binding" to it. Based on the findings of Nicholas Starkey that a second binding site is present in estrogen receptor β [62], it is possible that DIM binds to this alternate binding site and activates ER, but this binding does not allosterically influence the binding affinity of E2 in the orthosteric site in an appreciable way. In this case, DIM is not a competitor with E2 in binding to ER. To test this hypothesis, a binding assay with radio-labeled DIM will be useful to directly show the binding of DIM to ER. After confirming the binding of DIM to ER, an x-ray crystallography study would provide details of how DIM binds to the ER structure.

Another possible mechanism of how DIM inhibits prostate cancer may be by modulating the expression of ER. As described earlier, some labs have reported that DIM decreased expression of ERα mRNA [163, 164]. ERα expression increases in prostate cancer (PCa) epithelial cells, with very little ERα expressed in normal prostate and about 80% PCa epithelial cells expressing ERα. In contrast, ERβ expression declines in prostate cancer, after previously being expressed in normal prostate epithelial, but only 10% or less PCa cells being ERβ positive [195-197]. This, together with our published ERKO study in TRAMP mice, leads to the hypothesis that DIM might work by reducing ERα, which promotes cancer. A future experiment would measure both ERα and ERβ expression after DIM treatment in prostate cancer cell lines using qRT-PCR and western blot.

In Chapter II, we also showed that DIM inhibited growth of prostate cancer cell lines expressing ERβ, while inducing the growth of MCF7, a breast cancer cell line expressing only ERα, indicating ERβ was involved in the growth inhibitory effect of DIM. It would be nice to confirm the necessary involvement of ERβ in DIM function in a prostate cancer cell line. Since we could not find a prostate cancer cell line expressing only ERα, ERβ siRNA or Crispr-Cas9 could be used to knock out the expression of ERβ in those cells and then determine if the growth inhibition by DIM will decreased. Actually, we did try to use Crispr-Cas9 to knock out ER, but somehow, we could not get the transfection work in our prostate cancer cell line. Thus we used the ERα-only MCF7 cells.

In Chapter III, we had very nice data showing that DIM could prevent PDC in TRAMP mouse model dose-dependently. An immunohistochemistry (IHC) assay can be done to measure the possible change of ER expression after DIM treatment and to see if it will match the changes in the TRAMP-C2 cell line.

A follow up *in vivo* experiment will be testing DIM in the double transgenic ERKO TRAMP mice. Because of the "taste" issue and the resulting weight loss that we had in the Chapter III study, we need to consider a better way to dose the mice. We can gavage feed the mice, which controls the dosage more accurate. However, since we usually have very large sample size, it is very time consuming. Additionally, we don't want to stress the study mice. Another way can be adding sugar or other component to the diet to make mice like it. A small experiment should be pre-run after the diet was made to make sure that mice like and will eat the diet.

In the double transgenic ERKO TRAMP mice study, the cancer incidence will be examined to characterize the role of ER in DIM's prevention of prostate cancer. Another thing that may be of interest to look at is the body weight difference between the DIM treatment and control groups. There is always a big concern about obesity in developed/western countries, since people usually have a different life style and have a

much higher fat content in their diet when compared with people from Asian countries. We and another group showed that DIM caused weight loss when mice were on high fat diet [193]. We explained the initial weight loss observed in the early time of our study, which was because of mice disliking the taste. However, we didn't eliminate the effect DIM might have on body weight after long term consumption. ERβ is reported to alleviate obesity [194]. By comparing the mouse body weight among groups, we can decide whether and/or which ER might mediate DIM's repression of weight gain and perhaps its ability to prevent obesity.

Aside from what we have found with our data or in the literature, an RNAseq analysis in a DIM-treated prostate cancer cell line will be useful in discovering new DIM targets and help confirm our hypotheses on DIM's effects on both inhibiting cancer and preventing obesity/weight gain.

APPENDIX- I

INHIBITION OF ADVANCED PROSTATE CANCER BY DIETARY CHOLESTEROL IN TRAMP MOUSE MODEL

Overview

The role of cholesterol in cancer development is controversial in epidemiologic research. Here we summarize data from five TRAMP studies (2012-2017) in our lab. TRAMP mice were fed on three different diets with various fat and cholesterol contents: the low fat no cholesterol (LFNC) diet containing 7% fat but no cholesterol, the high fat low cholesterol (HFLC) diet containing 21% fat and 0.06% cholesterol, and the high fat high cholesterol (HFHC) diet containing 21% fat and 0.21% cholesterol. The overall PDC incidence of TRAMP mice fed on LFNC diet was 39%. Mice fed on HFLC diet had a significantly higher PDC incidence of 56%. However, adding an extra 0.15% cholesterol to the HFLC diet greatly decreased the PDC incidence compared to the HFLC diet, with only 33% PDC incidence in the HFHC diet. The extra added cholesterol in HFHC diet also reduced the overall cancer incidence of TRAMP mice when compared with mice on HFLC diet. Overall, our data suggested that dietary cholesterol had a prostate cancer protective effect in the TRAMP mouse model.

Introduction

The correlation between cholesterol and cancer development has been a long-term debatable question in prostate cancer research. Epidemiologic studies indicated that men with hypercholesterolemia had a higher risk for developing prostate cancer, and for men with dyslipidemia, a 10mg/dl increase in cholesterol could result in 9% increase in prostate cancer recurrence [198, 199]. A few additional studies suggested that cholesterol is only correlated with advanced prostate cancer. Low serum cholesterol reduced the risk for men to develop advanced prostate cancer, while high cholesterol increased the risk [128, 129, 200, 201]. Furthermore, statins, the cholesterol-lowing drug, are reported to prevent prostate cancer, as well as reduce cancer mortality [138-140, 202-206]. These together, suggest that cholesterol is positively associated with prostate cancer risk. However, it is not always the case. Recently, several other epidemiologic studies showed conflicting results, with low cholesterol increasing risk for prostate cancer and recurrence [132, 133, 207]. Even for the usually believed “bad” cholesterol, low-density lipoprotein (LDL), the correlation can be controversial, showing that prostate cancer patients with high levels of LDL cholesterol had much less cancer recurrence compared to those with low LDL levels [208]. The negative association between cholesterol and cancer risk was also observed in other cancer types, including lung, breast, pancreas and colorectal cancer [209-212].

With the contradictory results from various epidemiologic studies regarding the role of cholesterol in prostate cancer, more preclinical data are needed to support the result from epidemiologic studies. In this chapter, we summarized results from five TRAMP studies (2012-2017) on three different diets with various fat and cholesterol components, which suggest that dietary cholesterol might be protective for prostate cancer.

Materials and Methods

TRAMP mice

F1 C57/FVB hybrid male TRAMP mice were bred and housed as described in Chapter III.

Diet Formulations

Three diets with various fat and cholesterol content were used the study (Table AI-1A): 1) Low fat no cholesterol (LFNC) diet based on AIN93G (Figure AI-1A), 2) High fat high cholesterol (HFHC) western diet D12079B (Figure AI-1B) and 3) High fat low cholesterol (HFLC) diet, which is modified from the HFHC diet by removing the added cholesterol but leaving the same fat content (the same diet used in Chapter III). Diets were purchased from Research Diets, Inc (New Brunswick, NJ).

Statistical Analysis

All data was analyzed using Chi-squared test to determine significance.

Results

In order to characterize the effects of dietary fat and cholesterol on prostate cancer development, we collected data from the control groups of five TRAMP mice studies in our lab between 2012 to 2017 (Table AI-1B). We used three different control diets in those studies: the LFNC, HFHC and HFLC diets (Table AI-1A). Four studies used the LFNC diet, with their PDC incidence to be 39%, 44%, 37% and 35% respectively. No statistical difference was found among those studies (Table AI-2). The HFHC diet was used in only two studies. However, their PDC incidence was not consistent with each other. Statin study had 46% PDC, while Pectasol- c study had only 22%, which was statistical different than that in Statin study with p= 0.023 (Table AI-3). Three studies used the HFLC diet and their PDC incidence were 61%, 60% and 45%. Though there was lower PDC incidence in the Vaccine study, it was not statistically different from the other two (Table AI-4). Combining data from all studies together, PDC incidence in TRAMP mice fed with LFNC diet was 39%. Increasing fat content in the diet from 7% to 21% w/w (HFLC diet, also containing 0.06% w/w cholesterol, as cholesterol included in the milk fat) greatly increased the PDC incidence to 56% (p=0.007), suggesting that high fat diet could increase the risk of PDC. Surprisingly, feeding TRAMP mice with HFHC diet,

which was made by adding 0.15% w/w more cholesterol to the HFLC diet, made the PDC incidence drop back to 33% (p=0.001). There was no statistical difference between PDC incidence of the LFNC and HFHC diets (p=0.34). In addition, the overall cancer incidence (sum of WDC, MDC and PDC) of HFHC (79%) diet was also statistically lower than that of HFLC diet (92%, p=0.01) (Table AI-5). Taken together, our data indicated that cholesterol had a prostate cancer protective effect.

Discussion

While there are contradictory results in epidemiologic studies regarding the role of cholesterol in prostate cancer development, results of preclinical studies involving *in vitro* cell culture and *in vivo* animal models are much more consistent, showing that cholesterol positively correlates with prostate cancer development or cell proliferation [213-216]. In contrast to them, our data surprisingly shows, the cancer protective effect of dietary cholesterol in animal model.

When summarizing data from all five TRAMP studies, it was interesting to find that HFHC diet resulted in lower PDC and overall cancer incidence in TRAMP mice when compared to the HFLC diet, which shared the same fat content (21%) with HFHC diet, but lower cholesterol (0.06% vs. 0.21%). However, looking at cancer incidence of each diet separately, unlike the LFNC and HFLC diets, there was inconsistency of cancer

incidence in HFHC diet. The HFHC diet was tested only twice, in both the statin and pectasol-c study. Each of them had a PDC incidence of 46% and 22% and was statistically different with each other (Table AI-3). Since HFHC diet was not tested in parallel with HFLC diet in the pectasol-c study (Table AI-1B), the unrepeatable results from these two studies made it reluctant to combine them together. Additionally, the LFNC diet tested in pectasol-c study had a PDC incidence of 37%, compared to the HFHC diet in the same study being 22%, making this result less reliable. A future study testing HFHC in parallel with HFLC diet is needed to confirm the cancer protective effect of dietary cholesterol.

While majority of the epidemiologic studies focus on the serum cholesterol levels and prostate cancer development, one study reported relationship of dietary cholesterol intake and the risk of various cancers. In their study, high dietary cholesterol intake either increased or did not affect the risk of almost all cancers that they examined, including stomach cancer, breast cancer, lung cancer and others. The only exception was prostate cancer, which was inversely associated with dietary cholesterol intake [217]. Our result was consistent with the previous epidemiologic study showing increasing dietary cholesterol would decrease prostate cancer incidence.

Explanation for this cancer inhibitory effect of dietary cholesterol is unclear. One

possibility could be that increase of dietary cholesterol results in increase of serum cholesterol level, which then inhibits the *de novo* cholesterol synthesis within the prostate cancer cells through feedback inhibition. It is reported that androgen stimulates lipogenic gene expression in prostate cancer cells, such as the HMG-CoA reductase [218-220]. Thus, prostate, as an androgen responsive organ, may synthesize large amount of cholesterol, and presumably, some side products of cholesterol synthesis would be promoting prostate cancer development. We can test this hypothesis by using qRT-PCR and western blot to check the expressions of cholesterol uptake regulating LDL receptor (LDLR) and cholesterol synthesis enzymes in the prostate.

Table AI- 1: Summary of prospective studies and diets

A) Diets

Diet	Fat (w/w%)	Cholesterol (w/w%)	Included Studies (N)
Low fat no cholesterol (LFNC)	7%	0	4
High fat high cholesterol (HFHC)	21%	0.21%	2
High fat low cholesterol (HFLC)	21%	0.06%	3

B) Studies

Study	Included Diets
1. Statin	LFNC, HFLC, HFHC
2. Sutherlandia	LFNC
3. Pectasol- c	LFNC, HFHC
4. DIM	HFLC
5. Vaccine	LFNC, HFLC

A) LFNC Diet

Product # D10012G	gm%	kcal%
Protein	20.0	20.3
Carbohydrate	64.0	63.9
Fat	7.0	15.8
Total		100.0
kcal/gm	3.9	
Ingredient	**gm**	**kcal**
Casein, 30 Mesh	200	800
L-Cystine	3	12
Corn Starch	397	1590
Maltodextrin	132	528
Sucrose	100	400
Cellulose	50	0
Soybean Oil	70	630
t-Butylhydroquinone	0.014	0
Mineral Mix S10022G	35	0
Vitamin Mix V10037	10	40
Choline Bitartrate	2.5	0
Total	**1000**	**4000**

B) HFHC Diet

Product # D12079B	gm%	kcal%
Protein	20	17
Carbohydrate	50	43
Fat	21	41
Total		100
kcal/gm	4.7	
Ingredient	**gm**	**kcal**
Casein, 80 Mesh	195	780
DL-Methionine	3	12
Corn Starch	50	200
Maltodextrin 10	100	400
Sucrose	341	1364
Cellulose	50	0
Milk Fat, Anhydrous*	200	1800
Corn Oil	10	90
Mineral Mix S10001	35	0
Calcium Carbonate	4	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
Cholesterol, USP*	1.5	0
Ethoxyquin	0.04	0
Total	**1001.54**	**4686**

Figure AI- 1:Formulation of LFNC and HFHC diets

Table AI- 2: Cancer Incidence in TRAMP mice fed with LFNC diet

LFNC Diet								
Study	**N**	**Tumor Stage**						
		Non-cancer n (%)			**Cancer n (%)**			
		Normal	**Hyperplasia**	**PIN**	**WDC**	**MDC**	**PDC**
1. Statin	**38**	0	0	12 (32%)	11 (29%)	0	15 (39%)
2. Sutherlandia	**39**	0	0	9 (23%)	13 (33%)	0	17 (44%)
3. Pectasol- c	**51**	10 (20%)	0	0	22 (43%)	0	19 (37%)
5. Vaccine	**17**	2 (12%)	0	0	9 (53%)	0	6 (35%)
Summary	**145**	12 (8%)	0	21 (14%)	55 (38%)	0	57 (39%)

Table AI- 3: Cancer Incidence in TRAMP mice fed with HFHC diet

HFHC Diet								
Study	**N**	**Tumor Stage**						
		Non-cancer n (%)			**Cancer n (%)**			
		Normal	**Hyperplasia**	**PIN**	**WDC**	**MDC**	**PDC**	
1. Statin	**37**	0	0	14 (38%)	5 (14%)	1 (3%)	17 (46%)	
3. Pectasol-c	**45**	3 (7%)	0	0	32 (71%)	0	10 (22%)	
Summary	**82**	3 (4%)	0	14 (17%)	37 (45%)	1 (1%)	27 (33%)	

Table AI- 4: Cancer Incidence in TRAMP mice fed with HFLC diet

HFLC Diet							
Study	N	Tumor Stage					
		Non-cancer n (%)			Cancer n (%)		
		Normal	Hyperplasia	PIN	WDC	MDC	PDC
1. Statin	**41**	0	2 (5%)	6 (14.5%)	8 (19.5%)	0	25 (61%)
4. DIM	**40**	0	0	0	16 (40%)	0	24 (60%)
5. Vaccine	**29**	1 (3%)	0	0	15 (52%)	0	13 (45%)
Summary	**110**	1 (1%)	2 (2%)	6 (5%)	39 (35%)	0	62 (56%)

Table AI- 5: Effects of dietary fat and cholesterol on cancer incidence in TRAMP mice

Diets	N	Tumor Stage					
		Non-cancer n (%)			Cancer n (%)		
		Normal	Hyperplasia	PIN	WDC	MDC	PDC
LFNC	**145**	12 (8%)	0	21(14%)	55(38%)	0	57 (39%)
HFLC	**110**	1 (1%)	2 (2%)	6 (5%)	39 (35%)	0	62 (56%)
HFHC	**82**	3 (4%)	0	14 (17%)	37 (45%)	1 (1%)	27 (33%)

APPENDIX- II

EFFECTS OF PECTASOL-C ON PROSTATE CANCER IN THE TRAMP MOUSE PROSTATE CANCER MODEL

Below are the results from a previously finished pectasol-c (PeC) study in our lab. PeC were fed to TRAMP mice by either adding to the diet or dissolving in the water. A total of five groups of mice were involved in the PeC-diet study. One group of mice was fed with the casein control (LFNC) diet, while the remaining four were fed with western diets (HFHC) with or without 0.08%, 0.4% and 2% Pec (diet formulations in Figure AI-1). Mice in PeC-water study were on western diets as well, with control mice having acidified water, and an additional group having 1% PeC dissolved in the acidified water.

There were no clear effects of PeC on cancer incidence in either the diet or the water study. Increasing PeC in the diets showed a trend of decreasing PDC incidence of TRAMP mice, though not statistically significant, making us wonder whether PeC stimulated PDC or we were on the wrong side of the dose-response curve (hormesis effect). However, mice fed with western diet had a lower PDC incidence compared to mice fed with casein control (22% vs 37%). We doubt whether the two controls were switched thus the results need further confirm, but it is a possibility.

Table AII- 1: Effects of pectasol-c on prostate cancer incidence in TRAMP mice

Diets	N	Tumor Stage					
		Non-cancer n (%)			Cancer n (%)		
		Normal	Hyperplasia	PIN	WDC	MDC	PDC
Western Diet (HFHC)	45	3 (7%)	0	0	32 (71%)	0	10 (22%)
Casein Control (LFNC)	51	10 (20%)	0	0	22 (43%)	0	19 (37%)
0.08% Pectisol-C	43	5 (12%)	0	0	20 (47%)	0	18 (42%)
0.4% Pectisol-C	50	7 (14%)	0	0	24 (48%)	0	19 (38%)
2.0% Pectisol-C	47	5 (10.6%)	0	2 (4%)	24 (51%)	0	16 (34%)
Acid H_20 control	46	8 (18%)	0	1 (2%)	17 (38%)	0	19 (42%)
1.0% Pectisol-C in Acid H_20	45	6 (13%)	0	0	20 (44%)	0	20 (44%)

REFERENCES

1. *SEER Cancer Stat Facts: Prostate Cancer*. 2017; Available from: http://seer.cancer.gov/statfacts/html/prost.html.
2. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer Statistics, 2017.* CA Cancer J Clin, 2017. **67**(1): p. 7-30.
3. J., A., *The case of scirrhous of the prostate gland with corresponding affliction of the lymphatic glands in the lumbar region and in the pelvis.* Lancet, 1853.
4. Denmeade, S.R. and J.T. Isaacs, *A history of prostate cancer treatment.* Nat Rev Cancer, 2002. **2**(5): p. 389-96.
5. Cook, E.D. and A.C. Nelson, *Prostate cancer screening.* Curr Oncol Rep, 2011. **13**(1): p. 57-62.
6. Barry, M.J., *Clinical practice. Prostate-specific-antigen testing for early diagnosis of prostate cancer.* N Engl J Med, 2001. **344**(18): p. 1373-7.
7. Pinsky, P.F., et al., *Extended mortality results for prostate cancer screening in the PLCO trial with median follow-up of 15 years.* Cancer, 2017. **123**(4): p. 592-599.
8. Schroder, F.H., et al., *Screening and prostate cancer mortality: results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 years of follow-up.* Lancet, 2014. **384**(9959): p. 2027-35.
9. Schroder, F.H., et al., *Prostate-cancer mortality at 11 years of follow-up.* N Engl J Med, 2012. **366**(11): p. 981-90.
10. Tsodikov, A., et al., *Reconciling the Effects of Screening on Prostate Cancer Mortality in the ERSPC and PLCO Trials.* Ann Intern Med, 2017. **167**(7): p. 449-455.
11. Mostaghel, E.A. and P.S. Nelson, *Intracrine androgen metabolism in prostate cancer progression: mechanisms of castration resistance and therapeutic implications.* Best Pract Res Clin Endocrinol Metab, 2008. **22**(2): p. 243-58.
12. Presti, J.C., Jr., et al., *Multicenter, randomized, double-blind, placebo controlled study to investigate the effect of finasteride (MK-906) on stage D prostate cancer.* J Urol, 1992. **148**(4): p. 1201-4.
13. Fleshner, N.E. and J. Trachtenberg, *Treatment of advanced prostate cancer with the combination of finasteride plus flutamide: early results.* Eur Urol, 1993. **24 Suppl 2**: p. 106-12.
14. Thompson, I.M., et al., *The influence of finasteride on the development of prostate cancer.* N Engl J Med, 2003. **349**(3): p. 215-24.
15. Chen, Y., C.L. Sawyers, and H.I. Scher, *Targeting the androgen receptor pathway in prostate cancer.* Curr Opin Pharmacol, 2008. **8**(4): p. 440-8.

16. Vasaitis, T.S., R.D. Bruno, and V.C. Njar, *CYP17 inhibitors for prostate cancer therapy.* J Steroid Biochem Mol Biol, 2011. **125**(1-2): p. 23-31.
17. de Bono, J.S., et al., *Abiraterone and increased survival in metastatic prostate cancer.* N Engl J Med, 2011. **364**(21): p. 1995-2005.
18. Reid, A.H., et al., *CYP17 inhibition as a hormonal strategy for prostate cancer.* Nat Clin Pract Urol, 2008. **5**(11): p. 610-20.
19. Tran, C., et al., *Development of a second-generation antiandrogen for treatment of advanced prostate cancer.* Science, 2009. **324**(5928): p. 787-90.
20. Scher, H.I., et al., *Antitumour activity of MDV3100 in castration-resistant prostate cancer: a phase 1-2 study.* Lancet, 2010. **375**(9724): p. 1437-46.
21. Berruti, A., D. Generali, and M. Tampellini, *Enzalutamide in prostate cancer after chemotherapy.* N Engl J Med, 2012. **367**(25): p. 2448; author reply 2448-9.
22. Scher, H.I., et al., *Increased survival with enzalutamide in prostate cancer after chemotherapy.* N Engl J Med, 2012. **367**(13): p. 1187-97.
23. James, N.D., et al., *Addition of docetaxel, zoledronic acid, or both to first-line long-term hormone therapy in prostate cancer (STAMPEDE): survival results from an adaptive, multiarm, multistage, platform randomised controlled trial.* Lancet, 2016. **387**(10024): p. 1163-77.
24. Sweeney, C.J., et al., *Chemohormonal Therapy in Metastatic Hormone-Sensitive Prostate Cancer.* N Engl J Med, 2015. **373**(8): p. 737-46.
25. Kantoff, P.W., et al., *Sipuleucel-T immunotherapy for castration-resistant prostate cancer.* N Engl J Med, 2010. **363**(5): p. 411-22.
26. Sheikh, N.A., et al., *Sipuleucel-T immune parameters correlate with survival: an analysis of the randomized phase 3 clinical trials in men with castration-resistant prostate cancer.* Cancer Immunol Immunother, 2013. **62**(1): p. 137-47.
27. Small, E.J., et al., *Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer.* J Clin Oncol, 2006. **24**(19): p. 3089-94.
28. Higano, C.S., et al., *Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer.* Cancer, 2009. **115**(16): p. 3670-9.
29. Van Acker, H.H., et al., *Bisphosphonates for cancer treatment: Mechanisms of action and lessons from clinical trials.* Pharmacol Ther, 2016. **158**: p. 24-40.
30. Hegemann, M., et al., *Denosumab treatment in the management of patients with advanced prostate cancer: clinical evidence and experience.* Ther Adv Urol, 2017. **9**(3-4): p. 81-88.
31. Gingrich, J.R., et al., *Metastatic prostate cancer in a transgenic mouse.* Cancer

Res, 1996. **56**(18): p. 4096-102.
32. Rennie, P.S., et al., *Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene.* Mol Endocrinol, 1993. **7**(1): p. 23-36.
33. Watson, P.A., V.K. Arora, and C.L. Sawyers, *Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer.* Nat Rev Cancer, 2015. **15**(12): p. 701-11.
34. Gingrich, J.R., et al., *Androgen-independent prostate cancer progression in the TRAMP model.* Cancer Res, 1997. **57**(21): p. 4687-91.
35. Huggins, C. and P.J. Clark, *Quantitative Studies of Prostatic Secretion : Ii. The Effect of Castration and of Estrogen Injection on the Normal and on the Hyperplastic Prostate Glands of Dogs.* J Exp Med, 1940. **72**(6): p. 747-62.
36. Slusarz, A., et al., *Aggressive Prostate Cancer Is Prevented in ERalphaKO Mice and Stimulated in ERbetaKO TRAMP Mice.* Endocrinology, 2012.
37. Ellem, S.J. and G.P. Risbridger, *The dual, opposing roles of estrogen in the prostate.* Ann N Y Acad Sci, 2009. **1155**: p. 174-86.
38. Bonkhoff, H. and R. Berges, *The evolving role of oestrogens and their receptors in the development and progression of prostate cancer.* Eur Urol, 2009. **55**(3): p. 533-42.
39. Ricke, W.A., et al., *Prostatic hormonal carcinogenesis is mediated by in situ estrogen production and estrogen receptor alpha signaling.* FASEB J, 2008. **22**(5): p. 1512-20.
40. Steiner, M.S. and S. Raghow, *Antiestrogens and selective estrogen receptor modulators reduce prostate cancer risk.* World J Urol, 2003. **21**(1): p. 31-6.
41. Weihua, Z., et al., *An endocrine pathway in the prostate, ERbeta, AR, 5alpha-androstane-3beta,17beta-diol, and CYP7B1, regulates prostate growth.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13589-94.
42. Signoretti, S. and M. Loda, *Estrogen receptor beta in prostate cancer: brake pedal or accelerator?* Am J Pathol, 2001. **159**(1): p. 13-6.
43. Slusarz, A., et al., *Aggressive prostate cancer is prevented in ERalphaKO mice and stimulated in ERbetaKO TRAMP mice.* Endocrinology, 2012. **153**(9): p. 4160-70.
44. Omoto, Y., et al., *Estrogen receptor alpha and imprinting of the neonatal mouse ventral prostate by estrogen.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1484-9.
45. McEwan, I.J., *The Nuclear Receptor Superfamily at Thirty.* Methods Mol Biol, 2016. **1443**: p. 3-9.
46. Robinson-Rechavi, M., H. Escriva Garcia, and V. Laudet, *The nuclear receptor superfamily.* J Cell Sci, 2003. **116**(Pt 4): p. 585-6.

47. Jensen, E.V., *On the mechanism of estrogen action.* Perspect Biol Med, 1962. **6**: p. 47-59.
48. Kuiper, G.G., et al., *Cloning of a novel receptor expressed in rat prostate and ovary.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 5925-30.
49. Flouriot, G., et al., *Identification of a new isoform of the human estrogen receptor - alpha (hER - α) that is encoded by distinct transcripts and that is able to repress hER - α activation function 1.* The EMBO Journal, 2000. **19**(17): p. 4688-4700.
50. Denger, S., et al., *ERα Gene Expression in Human Primary Osteoblasts: Evidence for the Expression of Two Receptor Proteins.* Molecular Endocrinology, 2001. **15**(12): p. 2064-2077.
51. Wang, Z., et al., *A variant of estrogen receptor-{alpha}, hER-{alpha}36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 9063-8.
52. Heldring, N., et al., *Estrogen receptors: how do they signal and what are their targets.* Physiol Rev, 2007. **87**(3): p. 905-31.
53. Omarjee, S., et al., *The molecular mechanisms underlying the ERα-36-mediated signaling in breast cancer.* Oncogene, 2016. **36**: p. 2503.
54. Pietras, R.J., E.R. Levin, and C.M. Szego, *Estrogen receptors and cell signaling.* Science, 2005. **310**(5745): p. 51-3; author reply 51-3.
55. Shanle, E.K. and W. Xu, *Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action.* Chem Res Toxicol, 2011. **24**(1): p. 6-19.
56. Li, Y., et al., *Differential estrogenic actions of endocrine-disrupting chemicals bisphenol A, bisphenol AF, and zearalenone through estrogen receptor alpha and beta in vitro.* Environ Health Perspect, 2012. **120**(7): p. 1029-35.
57. Kerdivel, G., D. Habauzit, and F. Pakdel, *Assessment and molecular actions of endocrine-disrupting chemicals that interfere with estrogen receptor pathways.* Int J Endocrinol, 2013. **2013**: p. 501851.
58. McRobb, F.M., I. Kufareva, and R. Abagyan, *In silico identification and pharmacological evaluation of novel endocrine disrupting chemicals that act via the ligand-binding domain of the estrogen receptor alpha.* Toxicol Sci, 2014. **141**(1): p. 188-97.
59. Donoghue, L.J., et al., *Differential Activation of a Mouse Estrogen Receptor beta Isoform (mERbeta2) with Endocrine-Disrupting Chemicals (EDCs).* Environ Health Perspect, 2017. **125**(4): p. 634-642.
60. Sato, H., et al., *Oxysterol regulation of estrogen receptor alpha-mediated gene expression in a transcriptional activation assay system using HeLa cells.* Biosci Biotechnol Biochem, 2004. **68**(8): p. 1790-3.

61. Hirvonen, J., et al., *Transcriptional activity of estrogen-related receptor gamma (ERRgamma) is stimulated by the phytoestrogen equol.* J Steroid Biochem Mol Biol, 2011. **123**(1-2): p. 46-57.
62. Starkey, N.J.E., et al., *27-Hydroxycholesterol Is an Estrogen Receptor beta-Selective Negative Allosteric Modifier of 17beta-Estradiol Binding.* Endocrinology, 2018. **159**(5): p. 1972-1981.
63. McPherson, S.J., et al., *Estrogen receptor-beta activated apoptosis in benign hyperplasia and cancer of the prostate is androgen independent and TNFalpha mediated.* Proc Natl Acad Sci U S A, 2010. **107**(7): p. 3123-8.
64. Prins, G.S., et al., *Estrogen imprinting of the developing prostate gland is mediated through stromal estrogen receptor alpha: studies with alphaERKO and betaERKO mice.* Cancer Res, 2001. **61**(16): p. 6089-97.
65. Prins, G.S. and K.S. Korach, *The role of estrogens and estrogen receptors in normal prostate growth and disease.* Steroids, 2008. **73**(3): p. 233-44.
66. Bao, B.Y., et al., *Androgen receptor mediates the expression of UDP-glucuronosyltransferase 2 B15 and B17 genes.* Prostate, 2008. **68**(8): p. 839-48.
67. Mottet, N., et al., *Intermittent hormonal therapy in the treatment of metastatic prostate cancer: a randomized trial.* BJU Int, 2012. **110**(9): p. 1262-9.
68. Jang, J.W., et al., *Upfront androgen deprivation therapy with salvage radiation may improve biochemical outcomes in prostate cancer patients with post-prostatectomy rising PSA.* Int J Radiat Oncol Biol Phys, 2012. **83**(5): p. 1493-9.
69. Katzenellenbogen, B.S. and J.A. Katzenellenbogen, *Estrogen receptor transcription and transactivation: Estrogen receptor alpha and estrogen receptor beta: regulation by selective estrogen receptor modulators and importance in breast cancer.* Breast Cancer Res, 2000. **2**(5): p. 335-44.
70. Kollermann, J., et al., *Prognostic significance of disseminated tumor cells in the bone marrow of prostate cancer patients treated with neoadjuvant hormone treatment.* J Clin Oncol, 2008. **26**(30): p. 4928-33.
71. Robinson, D., et al., *PSA kinetics provide improved prediction of survival in metastatic hormone-refractory prostate cancer.* Urology, 2008. **72**(4): p. 903-7.
72. Pasquali, D., et al., *Loss of estrogen receptor beta expression in malignant human prostate cells in primary cultures and in prostate cancer tissues.* J Clin Endocrinol Metab, 2001. **86**(5): p. 2051-5.
73. Bonkhoff, H., et al., *Estrogen receptor expression in prostate cancer and premalignant prostatic lesions.* Am J Pathol, 1999. **155**(2): p. 641-7.
74. Nojima, D., et al., *CpG hypermethylation of the promoter region inactivates the*

estrogen receptor-beta gene in patients with prostate carcinoma. Cancer, 2001. **92**(8): p. 2076-83.
75. Barba, F.J., et al., *Bioavailability of Glucosinolates and Their Breakdown Products: Impact of Processing.* Front Nutr, 2016. **3**: p. 24.
76. Bjeldanes, L.F., et al., *Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol in vitro and in vivo: comparisons with 2,3,7,8-tetrachlorodibenzo-p-dioxin.* Proc Natl Acad Sci U S A, 1991. **88**(21): p. 9543-7.
77. Jellinck, P.H., et al., *Ah receptor binding properties of indole carbinols and induction of hepatic estradiol hydroxylation.* Biochem Pharmacol, 1993. **45**(5): p. 1129-36.
78. Li, Y., X. Li, and F.H. Sarkar, *Gene expression profiles of I3C- and DIM-treated PC3 human prostate cancer cells determined by cDNA microarray analysis.* J Nutr, 2003. **133**(4): p. 1011-9.
79. Mulvey, L., et al., *Interplay of genes regulated by estrogen and diindolylmethane in breast cancer cell lines.* Mol Med, 2007. **13**(1-2): p. 69-78.
80. Chen, I., S. Safe, and L. Bjeldanes, *Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells.* Biochem Pharmacol, 1996. **51**(8): p. 1069-76.
81. Degner, S.C., et al., *Targeting of aryl hydrocarbon receptor-mediated activation of cyclooxygenase-2 expression by the indole-3-carbinol metabolite 3,3'-diindolylmethane in breast cancer cells.* J Nutr, 2009. **139**(1): p. 26-32.
82. Licznerska, B.E., et al., *Modulation of CYP19 expression by cabbage juices and their active components: indole-3-carbinol and 3,3'-diindolylmethene in human breast epithelial cell lines.* Eur J Nutr, 2013. **52**(5): p. 1483-92.
83. Sanderson, J.T., et al., *2,3,7,8-Tetrachlorodibenzo-p-dioxin and diindolylmethanes differentially induce cytochrome P450 1A1, 1B1, and 19 in H295R human adrenocortical carcinoma cells.* Toxicol Sci, 2001. **61**(1): p. 40-8.
84. Bradlow, H.L., et al., *2-hydroxyestrone: the 'good' estrogen.* J Endocrinol, 1996. **150 Suppl**: p. S259-65.
85. Gupta, M., A. McDougal, and S. Safe, *Estrogenic and antiestrogenic activities of 16alpha- and 2-hydroxy metabolites of 17beta-estradiol in MCF-7 and T47D human breast cancer cells.* J Steroid Biochem Mol Biol, 1998. **67**(5-6): p. 413-9.
86. Fotsis, T., et al., *The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth.* Nature, 1994. **368**(6468): p. 237-9.
87. Klauber, N., et al., *Inhibition of angiogenesis and breast cancer in mice by the*

microtubule inhibitors 2-methoxyestradiol and taxol. Cancer Res, 1997. **57**(1): p. 81-6.
88. Lewis, J.S., et al., *Regulation of cell cycle and cyclins by 16alpha-hydroxyestrone in MCF-7 breast cancer cells.* J Mol Endocrinol, 2001. **27**(3): p. 293-307.
89. Telang, N.T., et al., *Induction by estrogen metabolite 16 alpha-hydroxyestrone of genotoxic damage and aberrant proliferation in mouse mammary epithelial cells.* J Natl Cancer Inst, 1992. **84**(8): p. 634-8.
90. Barba, M., et al., *Urinary estrogen metabolites and prostate cancer: a case-control study and meta-analysis.* J Exp Clin Cancer Res, 2009. **28**: p. 135.
91. Muti, P., et al., *Urinary estrogen metabolites and prostate cancer: a case-control study in the United States.* Cancer Causes Control, 2002. **13**(10): p. 947-55.
92. Rajoria, S., et al., *3,3'-diindolylmethane modulates estrogen metabolism in patients with thyroid proliferative disease: a pilot study.* Thyroid, 2011. **21**(3): p. 299-304.
93. Dalessandri, K.M., et al., *Pilot study: effect of 3,3'-diindolylmethane supplements on urinary hormone metabolites in postmenopausal women with a history of early-stage breast cancer.* Nutr Cancer, 2004. **50**(2): p. 161-7.
94. Leong, H., G.L. Firestone, and L.F. Bjeldanes, *Cytostatic effects of 3,3'-diindolylmethane in human endometrial cancer cells result from an estrogen receptor-mediated increase in transforming growth factor-alpha expression.* Carcinogenesis, 2001. **22**(11): p. 1809-17.
95. Leong, H., et al., *Potent ligand-independent estrogen receptor activation by 3,3'-diindolylmethane is mediated by cross talk between the protein kinase A and mitogen-activated protein kinase signaling pathways.* Mol Endocrinol, 2004. **18**(2): p. 291-302.
96. Marques, M., et al., *Low levels of 3,3'-diindolylmethane activate estrogen receptor alpha and induce proliferation of breast cancer cells in the absence of estradiol.* BMC Cancer, 2014. **14**: p. 524.
97. Riby, J.E., et al., *Ligand-independent activation of estrogen receptor function by 3, 3'-diindolylmethane in human breast cancer cells.* Biochem Pharmacol, 2000. **60**(2): p. 167-77.
98. Vivar, O.I., et al., *Selective activation of estrogen receptor-beta target genes by 3,3'-diindolylmethane.* Endocrinology, 2010. **151**(4): p. 1662-7.
99. Ohtake, F., et al., *Modulation of oestrogen receptor signalling by association with the activated dioxin receptor.* Nature, 2003. **423**(6939): p. 545-50.
100. Le, H.T., et al., *Plant-derived 3,3'-Diindolylmethane is a strong androgen antagonist in human prostate cancer cells.* J Biol Chem, 2003. **278**(23): p. 21136-45.
101. Bhuiyan, M.M., et al., *Down-regulation of androgen receptor by*

3,3'-diindolylmethane contributes to inhibition of cell proliferation and induction of apoptosis in both hormone-sensitive LNCaP and insensitive C4-2B prostate cancer cells. Cancer Res, 2006. **66**(20): p. 10064-72.
102. Hwang, C., et al., *Anti-androgenic activity of absorption-enhanced 3, 3'-diindolylmethane in prostatectomy patients.* Am J Transl Res, 2016. **8**(1): p. 166-76.
103. Li, Y. and F.H. Sarkar, *Role of BioResponse 3,3'-Diindolylmethane in the Treatment of Human Prostate Cancer: Clinical Experience.* Med Princ Pract, 2016. **25 Suppl 2**: p. 11-7.
104. Palomera-Sanchez, Z., et al., *The phytochemical 3,3'-diindolylmethane decreases expression of AR-controlled DNA damage repair genes through repressive chromatin modifications and is associated with DNA damage in prostate cancer cells.* J Nutr Biochem, 2017. **47**: p. 113-119.
105. Fan, S., et al., *Low concentrations of diindolylmethane, a metabolite of indole-3-carbinol, protect against oxidative stress in a BRCA1-dependent manner.* Cancer Res, 2009. **69**(15): p. 6083-91.
106. Ernst, I.M., et al., *3,3'-Diindolylmethane but not indole-3-carbinol activates Nrf2 and induces Nrf2 target gene expression in cultured murine fibroblasts.* Free Radic Res, 2011. **45**(8): p. 941-9.
107. Saw, C.L., et al., *Pharmacodynamics of dietary phytochemical indoles I3C and DIM: Induction of Nrf2-mediated phase II drug metabolizing and antioxidant genes and synergism with isothiocyanates.* Biopharm Drug Dispos, 2011. **32**(5): p. 289-300.
108. Lo, R. and J. Matthews, *The aryl hydrocarbon receptor and estrogen receptor alpha differentially modulate nuclear factor erythroid-2-related factor 2 transactivation in MCF-7 breast cancer cells.* Toxicol Appl Pharmacol, 2013. **270**(2): p. 139-48.
109. Wu, T.Y., et al., *Epigenetic modifications of Nrf2 by 3,3'-diindolylmethane in vitro in TRAMP C1 cell line and in vivo TRAMP prostate tumors.* AAPS J, 2013. **15**(3): p. 864-74.
110. Yao, Z., et al., *3,3'-Diindolymethane ameliorates adriamycin-induced cardiac fibrosis via activation of a BRCA1-dependent anti-oxidant pathway.* Pharmacol Res, 2013. **70**(1): p. 139-46.
111. Fuentes, F., X. Paredes-Gonzalez, and A.N. Kong, *Dietary Glucosinolates Sulforaphane, Phenethyl Isothiocyanate, Indole-3-Carbinol/3,3'-Diindolylmethane: Anti-Oxidative Stress/Inflammation, Nrf2, Epigenetics/Epigenomics and In Vivo Cancer Chemopreventive Efficacy.* Curr Pharmacol Rep, 2015. **1**(3): p. 179-196.
112. Lu, L., et al., *3,3'-diindolylmethane mitigates total body irradiation-induced*

hematopoietic injury in mice. Free Radic Biol Med, 2016. **99**: p. 463-471.
113. Hajra, S., et al., *Attenuation of doxorubicin-induced cardiotoxicity and genotoxicity by an indole-based natural compound 3,3'-diindolylmethane (DIM) through activation of Nrf2/ARE signaling pathways and inhibiting apoptosis.* Free Radic Res, 2017. **51**(9-10): p. 812-827.
114. Ansell, P.J., et al., *Repression of cancer protective genes by 17beta-estradiol: ligand-dependent interaction between human Nrf2 and estrogen receptor alpha.* Mol Cell Endocrinol, 2005. **243**(1-2): p. 27-34.
115. Montano, M.M., et al., *Transcriptional regulation by the estrogen receptor of antioxidative stress enzymes and its functional implications.* Oncogene, 2004. **23**(14): p. 2442-53.
116. MacLennan, G.T., et al., *The influence of chronic inflammation in prostatic carcinogenesis: a 5-year followup study.* J Urol, 2006. **176**(3): p. 1012-6.
117. Kazma, R., et al., *Association of the innate immunity and inflammation pathway with advanced prostate cancer risk.* PLoS One, 2012. **7**(12): p. e51680.
118. Sfanos, K.S. and A.M. De Marzo, *Prostate cancer and inflammation: the evidence.* Histopathology, 2012. **60**(1): p. 199-215.
119. Fujii, T., et al., *Immunohistochemical analysis of inflammatory cells in benign and precancerous lesions and carcinoma of the prostate.* Pathobiology, 2013. **80**(3): p. 119-26.
120. Gucalp, A., et al., *Incidence of periprostatic white adipose tissue inflammation in men with prostate cancer.* Journal of Clinical Oncology, 2017. **35**(6_suppl): p. 63-63.
121. Kim, E.J., et al., *3,3'-diindolylmethane suppresses 12-O-tetradecanoylphorbol-13-acetate-induced inflammation and tumor promotion in mouse skin via the downregulation of inflammatory mediators.* Mol Carcinog, 2010. **49**(7): p. 672-83.
122. Ghisletti, S., et al., *17beta-estradiol inhibits inflammatory gene expression by controlling NF-kappaB intracellular localization.* Mol Cell Biol, 2005. **25**(8): p. 2957-68.
123. Galien, R. and T. Garcia, *Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-kappaB site.* Nucleic Acids Res, 1997. **25**(12): p. 2424-9.
124. Nettles, K.W., et al., *CBP Is a dosage-dependent regulator of nuclear factor-kappaB suppression by the estrogen receptor.* Mol Endocrinol, 2008. **22**(2): p. 263-72.
125. Sas, L., et al., *The interaction between ER and NFkappaB in resistance to endocrine therapy.* Breast Cancer Res, 2012. **14**(4): p. 212.
126. Mak, P., et al., *ERbeta regulation of NF-kB activation in prostate cancer is*

mediated by HIF-1. Oncotarget, 2015. **6**(37): p. 40247-54.
127. Torre, L.A., et al., *Global cancer statistics, 2012.* CA Cancer J Clin, 2015. **65**(2): p. 87-108.
128. Mondul, A.M., et al., *Association between plasma total cholesterol concentration and incident prostate cancer in the CLUE II cohort.* Cancer Causes Control, 2010. **21**(1): p. 61-8.
129. Platz, E.A., et al., *Men with low serum cholesterol have a lower risk of high-grade prostate cancer in the placebo arm of the prostate cancer prevention trial.* Cancer Epidemiol Biomarkers Prev, 2009. **18**(11): p. 2807-13.
130. Shafique, K., et al., *Cholesterol and the risk of grade-specific prostate cancer incidence: evidence from two large prospective cohort studies with up to 37 years' follow up.* BMC Cancer, 2012. **12**: p. 25.
131. YuPeng, L., et al., *Cholesterol Levels in Blood and the Risk of Prostate Cancer: A Meta-analysis of 14 Prospective Studies.* Cancer Epidemiol Biomarkers Prev, 2015. **24**(7): p. 1086-93.
132. Ohno, Y., et al., *Association between preoperative serum total cholesterol level and biochemical recurrence in prostate cancer patients who underwent radical prostatectomy.* Mol Clin Oncol, 2016. **4**(6): p. 1073-1077.
133. Heir, T., et al., *Cholesterol and prostate cancer risk: a long-term prospective cohort study.* BMC Cancer, 2016. **16**: p. 643.
134. Wong, W.W., et al., *HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis.* Leukemia, 2002. **16**(4): p. 508-19.
135. Istvan, E.S. and J. Deisenhofer, *Structural mechanism for statin inhibition of HMG-CoA reductase.* Science, 2001. **292**(5519): p. 1160-4.
136. McTaggart, F., et al., *Preclinical and clinical pharmacology of Rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor.* Am J Cardiol, 2001. **87**(5A): p. 28B-32B.
137. Holdgate, G.A., W.H. Ward, and F. McTaggart, *Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin.* Biochem Soc Trans, 2003. **31**(Pt 3): p. 528-31.
138. Shannon, J., et al., *Statins and prostate cancer risk: a case-control study.* Am J Epidemiol, 2005. **162**(4): p. 318-25.
139. Platz, E.A., et al., *Statin drugs and risk of advanced prostate cancer.* J Natl Cancer Inst, 2006. **98**(24): p. 1819-25.
140. Jacobs, E.J., et al., *Cholesterol-lowering drugs and advanced prostate cancer incidence in a large U.S. cohort.* Cancer Epidemiol Biomarkers Prev, 2007. **16**(11): p. 2213-7.

141. Ahern, T.P., et al., *Statin prescriptions and breast cancer recurrence risk: a Danish nationwide prospective cohort study.* J Natl Cancer Inst, 2011. **103**(19): p. 1461-8.
142. Murtola, T.J., et al., *Statins and prostate cancer prevention: where we are now, and future directions.* Nat Clin Pract Urol, 2008. **5**(7): p. 376-87.
143. Yamazaki, M., et al., *Tissue-selective uptake of pravastatin in rats: contribution of a specific carrier-mediated uptake system.* Biopharm Drug Dispos, 1996. **17**(9): p. 775-89.
144. Nakai, D., et al., *Human liver-specific organic anion transporter, LST-1, mediates uptake of pravastatin by human hepatocytes.* J Pharmacol Exp Ther, 2001. **297**(3): p. 861-7.
145. Yaşar, P., et al., *Molecular mechanism of estrogen-estrogen receptor signaling.* Reproductive Medicine and Biology, 2017. **16**(1): p. 4-20.
146. Ho, C.K. and F.K. Habib, *Estrogen and androgen signaling in the pathogenesis of BPH.* Nat Rev Urol, 2011. **8**(1): p. 29-41.
147. A., S., C. YW., and K. A, *Drug Discovery from Plants*, in *Bioactive Molecules and Medicinal Plants*, R. K. and M. J., Editors. 2008, Springer, Berlin, Heidelberg.
148. Balunas, M.J. and A.D. Kinghorn, *Drug discovery from medicinal plants.* Life Sci, 2005. **78**(5): p. 431-41.
149. Kirsh, V.A., et al., *Prospective study of fruit and vegetable intake and risk of prostate cancer.* J Natl Cancer Inst, 2007. **99**(15): p. 1200-9.
150. Kolonel, L.N., et al., *Vegetables, fruits, legumes and prostate cancer: a multiethnic case-control study.* Cancer Epidemiol Biomarkers Prev, 2000. **9**(8): p. 795-804.
151. Richman, E.L., P.R. Carroll, and J.M. Chan, *Vegetable and fruit intake after diagnosis and risk of prostate cancer progression.* Int J Cancer, 2012. **131**(1): p. 201-10.
152. Sarkar, F.H. and Y. Li, *Indole-3-carbinol and prostate cancer.* J Nutr, 2004. **134**(12 Suppl): p. 3493S-3498S.
153. Chinni, S.R., et al., *Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells.* Oncogene, 2001. **20**(23): p. 2927-36.
154. Green, S., et al., *Cloning of the human oestrogen receptor cDNA.* J Steroid Biochem, 1986. **24**(1): p. 77-83.
155. Greene, G.L., et al., *Sequence and expression of human estrogen receptor complementary DNA.* Science, 1986. **231**(4742): p. 1150-4.
156. Kumar, V., et al., *Localisation of the oestradiol-binding and putative DNA-binding domains of the human oestrogen receptor.* EMBO J, 1986. **5**(9): p. 2231-6.

157. Boonyaratanakornkit, V. and D.P. Edwards, *Receptor mechanisms of rapid extranuclear signalling initiated by steroid hormones.* Essays Biochem, 2004. **40**: p. 105-20.
158. Levin, E.R., *Integration of the extranuclear and nuclear actions of estrogen.* Mol Endocrinol, 2005. **19**(8): p. 1951-9.
159. Shenouda, N.S., et al., *Phytoestrogens in common herbs regulate prostate cancer cell growth in vitro.* Nutr Cancer, 2004. **49**(2): p. 200-8.
160. Yin, X.F., et al., *A selective aryl hydrocarbon receptor modulator 3,3'-Diindolylmethane inhibits gastric cancer cell growth.* J Exp Clin Cancer Res, 2012. **31**: p. 46.
161. Rzemieniec, J., et al., *Selective Aryl Hydrocarbon Receptor Modulator 3,3'-Diindolylmethane Impairs AhR and ARNT Signaling and Protects Mouse Neuronal Cells Against Hypoxia.* Mol Neurobiol, 2016. **53**(8): p. 5591-606.
162. Lau, K.M., et al., *Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation.* Cancer Res, 2000. **60**(12): p. 3175-82.
163. Wang, T.T., et al., *Estrogen receptor alpha as a target for indole-3-carbinol.* J Nutr Biochem, 2006. **17**(10): p. 659-64.
164. Aksu, E.H., et al., *3,3 diindolylmethane leads to apoptosis, decreases sperm quality, affects blood estradiol 17 beta and testosterone, oestrogen (alpha and beta) and androgen receptor levels in the reproductive system in male rats.* Andrologia, 2016. **48**(10): p. 1155-1165.
165. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2018.* CA Cancer J Clin, 2018. **68**(1): p. 7-30.
166. Society, A.C., *Cancer Facts & Figures 2018.* American Cancer Society, 2018.
167. ; Available from: https://clinicaltrials.gov/ct2/results?term=Diindolylmethane.
168. Thomson, C.A., et al., *A randomized, placebo-controlled trial of diindolylmethane for breast cancer biomarker modulation in patients taking tamoxifen.* Breast Cancer Res Treat, 2017. **165**(1): p. 97-107.
169. Gee, J.R., et al., *Phase Ib placebo-controlled, tissue biomarker trial of diindolylmethane (BR-DIMNG) in patients with prostate cancer who are undergoing prostatectomy.* Eur J Cancer Prev, 2016. **25**(4): p. 312-20.
170. Castanon, A., et al., *Effect of diindolylmethane supplementation on low-grade cervical cytological abnormalities: double-blind, randomised, controlled trial.* Br J Cancer, 2012. **106**(1): p. 45-52.
171. Paltsev, M., et al., *First results of the double-blind randomized placebo-controlled multicenter clinical trial of DIM-based therapy designed as personalized approach to reverse prostatic intraepithelial neoplasia (PIN).*

EPMA J, 2016. **7**: p. 5.
172. Fujioka, N., et al., *Harnessing the Power of Cruciferous Vegetables: Developing a Biomarker for Brassica Vegetable Consumption Using Urinary 3,3'-Diindolylmethane.* Cancer Prev Res (Phila), 2016. **9**(10): p. 788-793.
173. Kotsopoulos, J., et al., *BRCA1 mRNA levels following a 4-6-week intervention with oral 3,3'-diindolylmethane.* Br J Cancer, 2014. **111**(7): p. 1269-74.
174. Paltsev, M., et al., *Comparative preclinical pharmacokinetics study of 3,3'-diindolylmethane formulations: is personalized treatment and targeted chemoprevention in the horizon?* EPMA J, 2013. **4**(1): p. 25.
175. Heath, E.I., et al., *A phase I dose-escalation study of oral BR-DIM (BioResponse 3,3'- Diindolylmethane) in castrate-resistant, non-metastatic prostate cancer.* Am J Transl Res, 2010. **2**(4): p. 402-11.
176. Del Priore, G., et al., *Oral diindolylmethane (DIM): pilot evaluation of a nonsurgical treatment for cervical dysplasia.* Gynecol Oncol, 2010. **116**(3): p. 464-7.
177. Reed, G.A., et al., *Single-dose pharmacokinetics and tolerability of absorption-enhanced 3,3'-diindolylmethane in healthy subjects.* Cancer Epidemiol Biomarkers Prev, 2008. **17**(10): p. 2619-24.
178. Reed, G.A., et al., *Single-dose and multiple-dose administration of indole-3-carbinol to women: pharmacokinetics based on 3,3'-diindolylmethane.* Cancer Epidemiol Biomarkers Prev, 2006. **15**(12): p. 2477-81.
179. Cho, H.J., et al., *3,3'-Diindolylmethane inhibits prostate cancer development in the transgenic adenocarcinoma mouse prostate model.* Mol Carcinog, 2011. **50**(2): p. 100-12.
180. Fares, F., et al., *The potential efficacy of 3,3'-diindolylmethane in prevention of prostate cancer development.* Eur J Cancer Prev, 2010. **19**(3): p. 199-203.
181. Nachshon-Kedmi, M., F.A. Fares, and S. Yannai, *Therapeutic activity of 3,3'-diindolylmethane on prostate cancer in an in vivo model.* Prostate, 2004. **61**(2): p. 153-60.
182. Bayer, J., et al., *Linear and inverted U-shaped dose-response functions describe estrogen effects on hippocampal activity in young women.* Nat Commun, 2018. **9**(1): p. 1220.
183. Calabrese, E.J., *Estrogen and related compounds: biphasic dose responses.* Crit Rev Toxicol, 2001. **31**(4-5): p. 503-15.
184. Calabrese, E.J. and L.A. Baldwin, *Hormesis: U-shaped dose responses and their centrality in toxicology.* Trends Pharmacol Sci, 2001. **22**(6): p. 285-91.
185. Anderton, M.J., et al., *Physiological modeling of formulated and crystalline 3,3'-diindolylmethane pharmacokinetics following oral administration in mice.* Drug Metab Dispos, 2004. **32**(6): p. 632-8.

186. Shenouda, N.S., et al., *Phytosterol Pygeum africanum regulates prostate cancer in vitro and in vivo.* Endocrine, 2007. **31**(1): p. 72-81.
187. Toyohira, Y., et al., *Stimulatory effects of the soy phytoestrogen genistein on noradrenaline transporter and serotonin transporter activity.* Mol Nutr Food Res, 2010. **54**(4): p. 516-24.
188. Cao, Y. and E. Giovannucci, *Obesity and Prostate Cancer*, in *Obesity and Cancer*, T. Pischon and K. Nimptsch, Editors. 2016, Springer International Publishing: Cham. p. 137-153.
189. Buschemeyer, W.C., 3rd and S.J. Freedland, *Obesity and prostate cancer: epidemiology and clinical implications.* Eur Urol, 2007. **52**(2): p. 331-43.
190. Shimizu, H., R.K. Ross, and L. Bernstein, *Possible underestimation of the incidence rate of prostate cancer in Japan.* Jpn J Cancer Res, 1991. **82**(5): p. 483-5.
191. Klassen, A.C. and E.A. Platz, *What can geography tell us about prostate cancer?* Am J Prev Med, 2006. **30**(2 Suppl): p. S7-15.
192. Moore, R.W., et al., *2,3,7,8-Tetrachlorodibenzo-p-dioxin has both pro-carcinogenic and anti-carcinogenic effects on neuroendocrine prostate carcinoma formation in TRAMP mice.* Toxicol Appl Pharmacol, 2016. **305**: p. 242-249.
193. Yang, H., et al., *3,3'-Diindolylmethane suppresses high-fat diet-induced obesity through inhibiting adipogenesis of pre-adipocytes by targeting USP2 activity.* Mol Nutr Food Res, 2017. **61**(10).
194. Yepuru, M., et al., *Estrogen receptor-{beta}-selective ligands alleviate high-fat diet- and ovariectomy-induced obesity in mice.* J Biol Chem, 2010. **285**(41): p. 31292-303.
195. Yeh, C.R., et al., *Estrogen receptors in prostate development and cancer.* Am J Clin Exp Urol, 2014. **2**(2): p. 161-8.
196. Royuela, M., et al., *Estrogen receptors alpha and beta in the normal, hyperplastic and carcinomatous human prostate.* J Endocrinol, 2001. **168**(3): p. 447-54.
197. Leav, I., et al., *Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma.* Am J Pathol, 2001. **159**(1): p. 79-92.
198. Allott, E.H., et al., *Serum lipid profile and risk of prostate cancer recurrence: Results from the SEARCH database.* Cancer Epidemiol Biomarkers Prev, 2014. **23**(11): p. 2349-56.
199. Magura, L., et al., *Hypercholesterolemia and prostate cancer: a hospital-based case-control study.* Cancer Causes Control, 2008. **19**(10): p. 1259-66.
200. Solomon, K.R. and M.R. Freeman, *The complex interplay between cholesterol*

and prostate malignancy. Urol Clin North Am, 2011. **38**(3): p. 243-59.
201. Jamnagerwalla, J., et al., *Serum cholesterol and risk of high-grade prostate cancer: results from the REDUCE study.* Prostate Cancer Prostatic Dis, 2017.
202. Murtola, T.J., et al., *Cholesterol-lowering drugs and prostate cancer risk: a population-based case-control study.* Cancer Epidemiol Biomarkers Prev, 2007. **16**(11): p. 2226-32.
203. Graaf, M.R., et al., *The risk of cancer in users of statins.* J Clin Oncol, 2004. **22**(12): p. 2388-94.
204. Fowke, J.H. and S.S. Motley, *Statin use linked with a decrease in the conversion from high-grade prostatic intraepithelial neoplasia (HGPIN) to prostate cancer.* Carcinogenesis, 2018. **39**(6): p. 819-825.
205. Chen, Y.A., et al., *Simvastatin Therapy for Drug Repositioning to Reduce the Risk of Prostate Cancer Mortality in Patients With Hyperlipidemia.* Front Pharmacol, 2018. **9**: p. 225.
206. Pelton, K., M.R. Freeman, and K.R. Solomon, *Cholesterol and prostate cancer.* Curr Opin Pharmacol, 2012. **12**(6): p. 751-9.
207. Allott, E.H., et al., *Statin use, high cholesterol and prostate cancer progression; results from HCaP-NC.* Prostate, 2018. **78**(11): p. 857-864.
208. Wettstein, M.S., et al., *Prognostic Role of Preoperative Serum Lipid Levels in Patients Undergoing Radical Prostatectomy for Clinically Localized Prostate Cancer.* Prostate, 2017. **77**(5): p. 549-556.
209. Zhang, Y., et al., *Pretreatment direct bilirubin and total cholesterol are significant predictors of overall survival in advanced non-small-cell lung cancer patients with EGFR mutations.* Int J Cancer, 2017. **140**(7): p. 1645-1652.
210. Strohmaier, S., et al., *Total serum cholesterol and cancer incidence in the Metabolic syndrome and Cancer Project (Me-Can).* PLoS One, 2013. **8**(1): p. e54242.
211. Carter, P.R., et al., *Patients with a diagnosis of hyperlipidaemia have a reduced risk of developing breast cancer and lower mortality rates: a large retrospective longitudinal cohort study from the UK ACALM registry.* European Heart Journal, 2017. **38**(suppl_1): p. 644-645.
212. Forones, N.M., et al., *Cholesterolemia in colorectal cancer.* Hepatogastroenterology, 1998. **45**(23): p. 1531-4.
213. Llaverias, G., et al., *A Western-type diet accelerates tumor progression in an autochthonous mouse model of prostate cancer.* Am J Pathol, 2010. **177**(6): p. 3180-91.
214. Sun, Y., et al., *Cholesterol-induced activation of TRPM7 regulates cell proliferation, migration, and viability of human prostate cells.* Biochim Biophys Acta, 2014. **1843**(9): p. 1839-50.

215. Kim, J., et al., *The response of the prostate to circulating cholesterol: activating transcription factor 3 (ATF3) as a prominent node in a cholesterol-sensing network.* PLoS One, 2012. **7**(7): p. e39448.
216. Mostaghel, E.A., et al., *Impact of circulating cholesterol levels on growth and intratumoral androgen concentration of prostate tumors.* PLoS One, 2012. **7**(1): p. e30062.
217. Hu, J., et al., *Dietary cholesterol intake and cancer.* Ann Oncol, 2012. **23**(2): p. 491-500.
218. Heemers, H., et al., *Androgens stimulate lipogenic gene expression in prostate cancer cells by activation of the sterol regulatory element-binding protein cleavage activating protein/sterol regulatory element-binding protein pathway.* Mol Endocrinol, 2001. **15**(10): p. 1817-28.
219. Heemers, H., et al., *Androgens stimulate coordinated lipogenic gene expression in normal target tissues in vivo.* Mol Cell Endocrinol, 2003. **205**(1-2): p. 21-31.
220. Swinnen, J.V., et al., *Androgens, lipogenesis and prostate cancer.* J Steroid Biochem Mol Biol, 2004. **92**(4): p. 273-9.

VITA

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My dissertation research has resulted in the following papers:

1. 3,3'-diindolylmethane inhibits advanced prostate cancer in the TRAMP mouse model. *Yufei Li, Nathaniel W. Mahloch, Nicholas J.E. Starkey, Mónica Peña-Luna, Cynthia Besch-Williford, Kevin L. Fritsche, Dennis B. Lubahn* (Resubmitted)

2. 27-Hydroxycholesterol Is an Estrogen Receptor β-Selective Negative Allosteric Modifier of 17β-Estradiol Binding. *Starkey J.E Starkey, Yufei Li, Sara K. Drenkhahn-Weinaug, Jinghua Liu, Dennis B. Lubahn* (Endocrinology, 2018)

3. The Effects of Simvastatin and Genistein on Oxysterol Concentrations and Cancer Incidence in TRAMP Mice. *Sara Drenkhahn; Glenn Jackson; Ania Slusarz; Yufei Li; Yuan Lu; Amber Mann; Roxanne Galvin; Christal Huber; Jennifer Bogener; Jim Browning; Wiedmeyer, Rottinghaus, Fristche, Lubahn* (Under revision)