**Introduction to Application:**

We greatly appreciate the constructive comments of the reviewers of our original application. We are grateful that the reviewers noted that this work would have great impact on improving our understanding of the epigenetic modulations of oxidative stress and inflammation regulatory genes involved in prostate carcinogenesis and that our findings could lead to novel ways to prevent prostate inflammation and cancers. The summary statement notes some minor concerns and our responses are discussed below.

**Inhibition of inflammation mediated prostate cancer by CAM products through epigenetic modification.** Some reviewers noted that: **(1)** Epigenome was not well defined; **(2)** The CAM products used in this project have multiple targets in the body; **(3)** These agents would ever be of clinical value; and **(4)** The successful completion of this project may not change the concept on inflammation/epigenetics/prostate cancer. **Response: (1)** In order to provide a more comprehensive understanding of the epigenome modulation by our proposed CAM products, we have added regulatory RNA study, including microRNAs (miRNAs) and long noncoding RNAs (lincRNAs), in this resubmission (preliminary data regarding miRNA in **Fig. 12**). In addition, the Analytical Core had performed the study of TET1 involved in conversion of 5mC to 5hmC (Dr. Hart, manuscript submitted). **(2)** Our preliminary data show that the proposed CAM products have potent anti-inflammatory effects and would cause changes epigenetically in the expression/activity of epigenetic modifying enzymes and demethylation of oxidative stress and inflammation regulatory genes. This evidence suggests that epigenomic modulation could be an important mechanism in the anti-inflammation regulatory process, although they may not be the only one. In addition, the global epigenomic alteration by CAM products will be used as an end point, which will yield additional insights into the biological mechanisms and potential value of CAM products. **(3)** As whether these CAM products have clinical value, currently there are 19 ongoing clinical trials with I3C/DIM. For the triterpenoids, such as boswellic acid (BA), which is a naturally occurring pentacyclic triterpenoid, has been reported to possess antiretroviral, antimalarial, and anti-inflammatory properties in human. Several ongoing clinical trials with triterpenoid ursolic acid (UA) have been reported. **(4)** We know that some of these CAM products work experimentally in cells and in animals, and it is now very important for us to find the mechanism. If we achieve that, we will know what the targets are and then we can test these and other CAM compounds that affect these targets, alone or in combinations, for efficacy in the lab and in the clinic.

**Experimental approaches.** Some reviewers noted: **(1)** The techniques proposed in this proposal are not new; **(2)** LNCaP cells will not be used for xenografts, triterpenoid boswellic acid (BA) studied in Aim 3 was not tested in animal models, and dose-response would have been preferable to the single dosage design for each CAM product in animal models; **(3)** Rationale for using specific mouse models should be provided, including Pten and CYP1A-humanized mice; **(4)** Comparison of the data with those known epigenetic modulators are necessary. **Response: (1)** We believe the molecular approaches, including Chip-Seq, epigenomic modifying enzymes analysis are cutting edge technologies. More importantly, the answer as to how epigenomes of inflammatory and oxidative stress pathways can be modulated by CAM products resulting in prevention of inflammation related diseases will provide invaluable information of the outcome of CAM products. **(2)** As we proceed with our study, we will consider LNCaP xenograft model and additional dosage for *in vivo* study. We have deleted BA study in this resubmission. **(3)** Additional preliminary data showing the differences of mRNA levels of Nrf2, Nrf2 targeting gene NQO1, and inflammation genes COX2, iNOS and IL6 as well as the global methylation status in prostate tissues between control Pten(f/f) and Pten KO mice are now provided (**Fig. 14**). The preliminary data of CYP1A-humanized mice has been provided in **Fig. 15**. **(4)** In our *in vitro* study, DNMT inhibitor 5-azacytidine (5-aza) and histone inhibitor trichostatin A (TSA) will be used as positive control for evaluating the epigenetics modifying potential of CAM compounds in *in vitro* cell experiments; they will not be used in *in vivo* animal models due to their high toxicity. However some other natural non-toxic compounds, such as curcumin (currently supported by NCCAM R01AT007065), with epigenetics modifying potential as well as immunomodifying activity will be compared.

**Adjustment of investigators.** Over-commitment of Dr. Kong, the other key members who will conduct these studies appear to be weak, and the pathological evaluation should be the cutting edge of the research. **Response:** In this resubmission, Dr. Kong has relinquished the role of Analytical Core director. The members of this Project have been reorganized in which Dr. Zheng-Yuan Su will be the only postdoc with a PhD graduate student; both with 100% effort. The Pathology evaluation will be performed by the Analytical Core service with the addition of Dr. Michael Goedken, Director, Histopathology Core Facility, Office of Translational Science, Rutgers University.

**All the changes in the text are in *ITALIC FONT* with a line of indication on the right margin.**

**SPECIFIC AIMS:**

**Background:** Excessive and chronic inflammation can contribute to many acute and chronic diseases including autoimmune disease, neurological, cardiovascular disease, and cancer. Increasing evidence suggests that chronic inflammation is closely associated with epigenetic alterations, mediated by DNA and histone modifications, driving changes in the expression of many inflammatory genes, such as IL1R1, IL-1β, toll-like receptor 2, 15-lipoxygenase, cyclooxygenase-2, CXCL14, CCL25, CXCL6, IL13, IL17C and IL4R Importantly several classes of natural CAM products possess anti-inflammatory and epigenetic-modifying properties. Our Preliminary Studies show that the CpG methylation status of inflammatory genes, including IL18, Nos3, Col2a1, Hpgds, Ppara, Prkar1b, Itgb2, P2rx7, Klf2, Ptgs2, and Mif, were altered in TRAMP prostate tumor as compared to the control wild-type using MeDIP-seq technology. Similarly, in 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)-induced inflammation/high-grade PIN (HGP) in the prostate of CYP1A-humanized mice, inflammatory markers such as 8-oxo-dG, nitrotyrosine, COX-2, p-AKT, and PTEN were increased, and in mouse prostate specific Pten-/- high-grade PIN, inflammatory infiltrates were increased. Furthermore, indole-3-carbinol (I3C)/3,3'-diindolylmethane (DIM) from cruciferous vegetables and ursolic acid (UA), a triterpenoid, from medicinal plants, cranberry and blueberry, which posse potent anti-inflammatory activities in different types of cells, were found to possess epigenetics modifying activities. Despite these promising results, the epigenomic changes and the underlying epigenetics mechanisms of prostate inflammation and related diseases including prostate cancer (PCa) and how CAM products epigenetically modified the inflammatory epigenome leading to inhibition of these aberrant processes remains unknown. Better understanding of the epigenetics mechanisms of how CAM products inhibit inflammation and its related disease would open new avenue of approaches for the prevention and treatment of chronic inflammatory diseases including PCa in human.

**Overall Hypothesis:** Based on previous published reports and our preliminary studies, ***we hypothesize that chronic inflammatory processes would drive changes of inflammatory epigenome and CAM products would modify these inflammatory epigenomic alterations resulting in suppression of inflammation and its related diseases including cancer in the prostate.***

**Objectives:** Our objectives are to investigate the efficacy and epigenetic alterations by I3C/DIM and triterpenoid UA in preventing inflammation and inflammatory-related diseases in prostate tissues of Pten-null mice, PhiP-induced inflammation/HGP in CYP1A-humanized mice, and in TRAMP mice. Our long-term goal is to develop a safe and effective strategy of preventing chronic inflammation and its related diseases in humans using I3C/DIM and triterpenoids and to provide a new paradigm for epigenetic-based natural CAM anti-inflammatory modulators. To achieve these objectives, three Specific Aims are proposed:

**Aim 1:** To investigate the alterations of inflammatory and prostatic epigenetics in Pb-Cre/Pten(flox/flox) mice, PhIP induced CYP1A humanized mice and TRAMP mice, to evaluate the preventive efficacy of triterpenoid UA and I3C, and how these CAM products target the epigenomic regulation of anti-inflammation/oxidative stress genes.

**Aim 2:** To investigate the inflammation/apoptotic epigenome profiles in human VCaP PCa cells in NCr(-/-) nude mice, and to investigate the effect of UA and I3C on the anti-inflammatory/pro-apoptotic epigenome pathways.

**Aim 3:** To elucidate *in vitro* epigenetic mechanisms in the regulation of the inflammatory/oxidative stress and pro-apoptotic genes obtained from *in vivo* Aims 1 and 2 above by *UA and DIM* in mouse TRAMP C1 and Pten-CaP2 cell lines as well as human VCaP and LNCaP PCa cell lines.

**Impact:** The proposed investigation of *in vitro* and *in vivo* epigenomic studies in three different mouse models would provide a detail understanding of how epigenome modulations of inflammation/oxidative stress/pro-apoptotic pathways genes contribute to prostate diseases such as cancer, and the epigenetics modifications by CAM products I3C/DIM and triterpenoids in prevention of the disease. The success of the proposed study will further drive clinical trials of I3C/DIM and triterpenoids in prevention of chronic inflammation and related diseases in human including the PCa. The mechanistic exploration of the epigenetic modulation would provide potential biomarkers for future clinical trials and optimization of I3C/DIM and triterpenoids based anti-inflammation regimens against chronic inflammation and related diseases including cancer.

**RESEARCH STRATEGY:**

**(A) Background and Significance**

**A.1. Inflammation and prostate carcinogenesis:** Excessive and chronic inflammations contribute to many acute and chronic diseases including autoimmune disease, neurological, cardiovascular disease, and cancer. Increasing evidence suggests that chronic inflammation is closely associated with epigenetic alterations, mediated by DNA and histone modifications, driving changes in the expression of many inflammatory-related genes, such as IL1R1, IL-1β, toll-like receptor (TRL) 2, 15-lipoxygenase (15-LOX), cyclooxygenase-2 (COX2), CXCL14, CCL25, CXCL6, IL13, IL17C, and IL4R (1-8). Inflammation has been linked to approximately 20% of human cancers (9). The type and nature of inflammatory stimuli appear to be different for different cancer types, and thus, different mechanisms of tumor-promoting effects by inflammation have been proposed (10). In prostate carcinogenesis, growing evidence suggests a link between chronic or persistent inflammation and tumor development (11). Epidemiological data indicate that people diagnosed with chronic inflammatory prostatitis have an increased risk of developing prostate cancer (PCa) at a later age. Moreover, people receiving long-term treatment with NSAIDs have a reduced risk of developing PCa (12, 13).

**A.2. Inflammation related PCa and carcinogenesis models:** There is strong evidence that prostatic inflammation and oxidative stress contribute to human prostate carcinogenesis (11, 12, 14-17). Inflammation has increasingly been recognized as a critical component influencing PCa development and progression (16, 18). Areas of malignant glands in PCa are frequently juxtaposed to inflamed areas of prostate tissue, implying that either inflammation propagates the cancer or is responding to it (11). A recent hypothesis for inflammation-induced prostate carcinogenesis, that activated prostate epithelial stem cells acquire a survival advantage by expressing one or more of the same cytokines, such as IL-6. Establishment of one or more autocrine signaling loops expands these cells in the absence of inflammation as a potential first tumor development stage (19). Molecular changes such as overexpression of COX-2 and p16 and down-regulation of p27, p63, PTEN (phosphatase and tensin homolog deleted on chromosome 10), and NKX3.1 have been observed in human PCa (11). Glutathione *S* transferase P1 (GSTP1) is silenced through promoter methylation in subsets of cells resulting in enhanced oxidative stress (20) and related inflammation (21). Many inflammatory cytokines and chemokines promote tumor progression by converging on and stimulating the IKK2/NF-κB signaling axis (22). In animal model with constitutive active expression of IKK2 alone is insufficient in promoting prostate tumorigenesis, but when combined with heterozygous loss of Pten, IKK2 activation leads to an increase in tumor size, accompanied by increased inflammatory cytokines secreted from the stromal microenvironment of the prostate as a result of PTEN loss driving epithelial prostate tumor cells toward metastasis (23). The PTEN tumor suppressor gene is one of the most frequently mutated/deleted genes in various human cancers (24). It has been reported that in Prostate specific Pten null mice, there is increased expression of CXCL8/IL-8, a pro-inflammatory chemokine promoting tumorigenesis (25). PTEN deficiency in the epithelium shows a dose-dependent transformation of mouse prostate tissue, with low-grade prostatic intraepithelial neoplasia (PIN) in monoallelic deletions, and the lesions with ability to form invasive and eventually metastasizing carcinomas in full deletions (11, 26, 27). 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP), a dietary carcinogen which is one of the most abundant heterocyclic amines formed at high temperatures in cooked meat, has been shown to induce malignant lesions in rodents and primates (28-31). Exposure to PhIP in the diet induces carcinomas of the intestine, mammary gland and prostate in rats (32-35). In rat prostate, oral doses of PhIP cause inflammation, epithelial cell damage, PIN, and carcinomas (28, 29, 33, 34), but interestingly PhIP does not induce PCa in mice. A possible reason for the absence of PhIP-induced PCa in mice is due to the lack of an efficient enzyme system to activate PhIP. Initial metabolic activation of PhIP is carried out mainly by cytochrome P450 (CYP) enzymes, in particular CYP1A2 (36). Studies have shown that human CYP1A2 preferentially activates PhIP via N2-hydroxylation, which leads to the eventual formation of an ultimate carcinogen (36, 37). The mouse Cyp1a2, however, mainly catalyzes the 40-hydroxylation of PhIP, which is a detoxification pathway. It has been recently reported that in CYP1A-humanized (hCYP1A) mice, which express human CYP1A1 and CYP1A2 and are deficient in murine Cyp1a1 and Cyp1a2, a single oral dose of PhIP induced inflammation and PIN lesions in the prostate (37, 38). The TRAMP model exhibits many similarities to human PCa, including epithelial origin, progression from the PIN stage to adenocarcinoma, and metastasis by a transgene that is hormonally regulated by androgen (39-42), so that it serves as a suitable model (43). During tumorigenesis of TRAMP PCa, several inflammatory signaling pathways such as TLRs, TLR3 (44), MMP-2 (45), endoplasmic reticulum (ER) stress response, IL-6, interleukin 23p19 (IL-23p19), and TNF-α (46) have been implicated. Decreased serum levels of proinflammatory molecules (IL-6 and IL-11) after dietary tomato treatments in TRAMP mice are reported (47). Silencing of multiple genes by DNA methylation has been reported in TRAMP prostate tumors and cell lines derived from the TRAMP prostate tumors (48-50). Inhibition

of DNMT activity by 5-aza has been shown to prevent prostate tumorigenesis in TRAMP mice (51). In addition, stage and phenotype-specific CpG island methylation and DNMT expression have been well documented during PCa progression in TRAMP mice (52, 53). These published findings suggest the relevance of using this TRAMP system to investigate the role of epigenetic alterations in PCa.

**A.3. Inflammation and Oxidative Stress – cross-talk and interactions:** Many reports have shown that cross-talk and interactions between inflammation and oxidative stress in a number of physiological and pathological conditions occur (54-57). **Nuclear factor-erythroid 2-related factor 2 (Nrf2)** is a master regulator of anti-oxidative stress response (57-59), and many *in vitro* and *in vivo* studies show that Nrf2 is also essential for anti-inflammatory response. For instance, it has been reported that genetic or pharmacologic amplification of Nrf2 signaling inhibits acute inflammatory liver injury in mice (60). Many studies reported that enhanced NF-κB activation is observed in $Nrf2^{-/-}$ mice after stimuli such as traumatic brain injury (61), LPS and TNF-α (62). Mice lacking Nrf2 are more susceptible to AOM-DSS-induced colitis (63) and colorectal carcinogenesis (64). It was reported that NF-κB could directly repress Nrf2 signaling at the transcription level by competing against Nrf2 for transcription co-activator CREB binding protein (CBP) and NF-κB recruits histone deacetylase 3 (HDAC3) to cause local hypoacetylation to hamper Nrf2 signaling (65). A number of CAM phytochemicals activate Nrf2 signaling with a concomitant repression of pro-inflammatory pathways mediated by NF-κB signaling (66) and other inflammatory markers (67, 68). Importantly, loss of Nrf2 attenuates the anti-inflammatory effects of sulforaphane (SFN) and omega-3-fatty acids (69, 70). Hence, oxidative stress and inflammation processes appear to be tightly coupled, and the failure to stop these processes would result in genetic/epigenetic changes that drive the initiation of carcinogenesis (71-73).

**A.4. Targeting epigenome of inflammation and oxidative stress with CAM products as novel prostate disease prevention approach:** Several classes of natural CAM products have been shown to possess anti-inflammatory and epigenetic-modifying properties. It is postulated that changes in gene expression due to epigenetic modifications can be reversed by chemicals. Therefore, CAM products that target enzymes responsible for DNA methylation and/or histone modifications as well as microRNA (miRNA) can be useful to prevent prostate inflammation and related diseases. Some of histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) inhibitors have been approved for use in hematological malignancies and are currently in different phases of clinical trials (74, 75). DNA hypomethylating agents, 5-azacitdine (5-aza) and 5-aza-CdR (5-aza-2'-deoxycytidine; decitabine) have been shown effective in cancer treatments (76-79). Likewise, HDAC inhibitor (HDACi) suberoylanalide hydroxamic acid (SAHA) has been approved by the US FDA for therapy of T-cell lymphomas (80, 81) and others in phase I and phase I/II clinical trials (74, 78, 82-84). Serious side effects hindered the development of these compounds for long term prevention use (85-89). There is a growing body of evidence that many dietary phytochemicals preventive compounds could prevent diseases including cancer by modifications of epigenetic processes in the cells (90-92). Selenium, a potent cancer chemopreventive agent has been reported to be able to induce global DNA hypomethylation and re-activate epigenetically silenced tumor suppressor genes through inhibition of DNMT activity (93). Isothiocyanates (ITCs) such as SFN and phenethyl isothiocyanate (PEITC) from cruciferous vegetables; allyl compounds present in garlic, have all been reported as potent HDAC and/or DNMT inhibitors (94-96). Polyphenols, such as (–)-epigallocatechin 3-gallate (EGCG) from green tea and genistein from soybean have also been shown to inhibit DNMTs *in vitro* (91). Indole-3-carbinol (I3C) is a common phytochemical existing in cruciferous vegetables, and 3,3'-diindolylmethan (DIM) is an *in vivo* dimeric product of I3C. I3C and DIM have been reported to possess strong anti-inflammatory effects (97, 98). We and others found that, compared with I3C, DIM has stronger induction ability on transactivation of Nrf2 and expression of Nrf2 downstream antioxidant genes HO-1, GSTm2, UGT1A1, γGCS, SOD1, and NQO1 (99, 100). In this context, many natural dietary products which are cancer preventive phytochemicals have been shown to be electrophiles (eg, SFN, CUR and triterpenpoids) that can modify sulfhydryl (-SH) group of cysteine moiety of many proteins including Nrf2-Keap1 resulting in activation of Nrf2-ARE mediating induction of anti-oxidative stress/detoxifying genes leading to protection against oxidative stress and cellular injury (101). Coincidentally, many of these dietary phytochemicals that are electrophiles and Nrf2 activators have been shown to be epigenetics modifiers and the potential role of electrophiles' metabolites inhibiting HDACs has been implicated (102-107). DIM has attracted extensive attention for its cancer preventive potential in different mouse models of ovarian (108, 109), lung (110) and colon (111). To date, there are ten clinical trials being conducted in early stage of prostate, breast and cervical cancer patients. DIM regulates many genes that are important for the control of apoptosis, cell cycle, cell proliferation, signal transduction, and other cellular processes including STAT3 (signal transducer and activator of transcription 3), CDK inhibitors, Par-4 (prostate apoptosis response-4), EGFR, Akt, mTOR, and NF-κB (108, 109, 112, 113), dependence on tissues and cell lines. DIM treatment was also shown to be

associated with increased PTEN, miR-200 family expression and down regulation of MT1-MMP (114). In prostate, recently we found that DIM-supplemented diet attenuated incidence of tumorigenesis and metastasis in TRAMP mice. In addition, DIM activates the expression of Nrf2 through epigenetic modulation – DIM treatment reversed CpG methylation status of Nrf2 and suppressed the expression of DNMTs in this TRAMP mouse model and TRAMP C1 cells, a cell line derived from TRAMP prostate tumor (115). Some reports show that DIM possesses immunomodification and anti-cancer effects through regulating miRNA expression (116-119). In terms of triterpenoids, boswellic acid (BA) which is the major constituents of a gum resin derived from the plant *Boswellia serrata*, have been traditionally used in treatments for various inflammatory diseases and it has been shown to have antitumor effects in different types of tumor cells including colon, prostate, leukocytes, liver, and brain potentially via epigenetics mechanisms (120, 121). As an anti-inflammatory agent, BA has been shown interacting directly with IKK and suppressed NFκB-regulated genes (122, 123), and down-regulated the expression of COX-2, MMP-9, CXCR4, and VEGF in human pancreatic xenografts mouse model (124). Interestingly, recent epigenetic studies show that BA exerts anti-cancer effects in colorectal cancer cells by modulating expression of the let-7 and miR-200 microRNA family (121), and DNA methylation of several tumor suppressor genes (120). Ursolic acid (UA) is a natural pentacyclic triterpenoid carboxylic acid derived from medical herbs, fruits and vegetables (e.g. cranberry, blueberry, beets, mushrooms, *Rosemarinus officinalis*, *Eriobotrya japonica*, *Calluna vulgaris*, *Ocimum sanctum*, and *Eugenia jumbolana*) and exerts a wide range of biological activities, including anti-inflammatory and anticancer activities (125). UA has been reported to suppress the proliferation and induce apoptosis in a variety of tumor cells and to inhibit tumor promotion, metastasis and angiogenesis (37, 126). Recently, it was reported in TRAMP mice, UA exerts chemopreventive activity against PCa through down-regulated activation of various pro-inflammatory mediators including, NF-κB, STAT3, AKT, and IKKa/b phosphorylation correlating with the reduction in serum levels of TNF-α and IL-6 (126). UA also been shown to induce apoptosis in human glioblastoma U251 cells by a UA-triggered TGF-β1/miR-21/PDCD4 (programmed cell death 4) pathway (127).

## (B) Innovation

The present study will provide new insights into: (1) the epigenomics of inflammation and inflammation-related PCa; (2) epigenetic modifications by CAM products I3C/DIM and triterpenoid UA on the epigenome of inflammation/oxidative stress in prostate; and (3) efficacy of I3C/DIM and UA in altering the epigenetic events resulting in the prevention of prostate inflammation and carcinogenesis.

## (C) Approach

### C.1. Preliminary Studies:

***C.1.1. DIM re-activates Nrf2 and up-regulates Nrf2 downstream target genes through epigenetic modification in vitro and in vivo.*** Previously we have reported that 25-75 μM of DIM induced Nrf2-ARE-luciferase activity in HepG2-C8 cells after 24 h (**Fig. 1**) (99). To elucidate the potential molecular epigenetic mechanism, treating mouse PCa TRAMP C1 cells (128) with much lower doses (2.5 and 5 μM) of DIM for 5 days significantly suppressed the protein expression of DNMT1, DNMT3b, HDAC2, HDAC3, and HDAC4 (**Fig. 2**) (115).

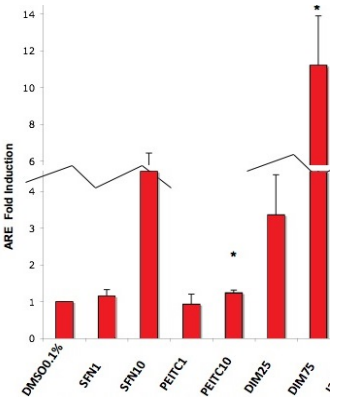


***Fig. 1.*** **DIM induces ARE-luciferase activity in HepG2-C8 cells.** The luciferase activity (ARE fold induction) was expressed as the fold induction over the vehicle treated cells.

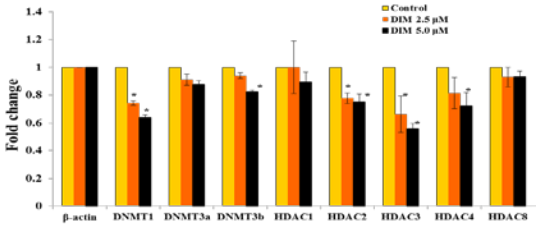


***Fig. 2.*** **DIM suppresses the protein expression of DNMT and HDAC in TRAMP C1 cells.** Bars represent mean fold change ± SD (normalized with β-actin and compared with control value). *$p$<0.05, significantly different from the control by Student's $t$ test.

Furthermore, bisulfite genomic sequencing (BGS) showed that DIM decreased the methylation status of the first 5 CpGs in the promoter region of Nrf2 gene in a dose-dependent manner (73.7% vs 55.8% methylation) (**Fig. 3**). MeDIP (methylated DNA immunoprecipitation) using anti-methyl-cytosine (mecyt) antibody (129) show that DIM reduced the methylated DNA bound by anti-mecyt antibody to the first 5 CpGs of Nrf2 gene promoter (115). The above results indicate that DIM treatment reduces the CpG methylation status of the Nrf2 gene promoter region in TRAMP C1 cells.

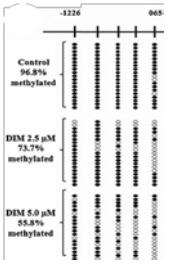


***Fig. 3.*** **Demethylation effect of DIM on Nrf2 promoter region in TRAMP C1 cells after 5 days treatment.** The methylation patterns of the first 5 CpGs of promoter Nrf2 gene in TRAMP C1 cells was performed using BGS. Filled and open circles indicate methylated and unmethylated CpGs, respectively.

Additionally, DIM significantly enhanced the mRNA expression of Nrf2 and Nrf2- target genes including NQO1, HO-1, GSTm1, and UGT1A1 in TRAMP C1 cells (**Fig. 4A**)(115). Corroborating the results of mRNA expressions, protein levels of Nrf2 and NQO1 were also significantly induced in TRAMP C1 cells treated with DIM (**Fig. 4B**) (115). These results suggest that DIM is able to modify the epigenetic status of CpG methylation of Nrf2, and restores mRNA and protein expression of Nrf2 and Nrf2-target gene in TRAMP C1 cells analogous to our recent finding with curcumin (130).

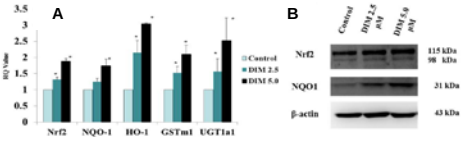


***Fig. 4.*** **Effect of DIM on mRNA (A) and protein (B) expression of Nrf2 and Nrf2 target genes in TRAMP C1 cells treated for 5 days.** Bars represent mean ± SD (normalized with β-actin and compared with control value). **p*<0.05.

In TRAMP mice, our Preliminary Study shows that DIM significantly decreased 5-methylcytidine IHC staining of the prostate tissue in DIM treated group (115). DIM significantly lowered methylation of the first 5 CpGs of Nrf2 (37.6% methylation) versus the control group (98% methylation) (**Fig. 5A**) [We have reported that the first 5 CpGs on the Nrf2 gene promoter region are hypermethylated *in vivo* TRAMP prostate tumors and *in vitro* TRAMP C1 cells but not in normal prostate tissues (128)]. These results suggest that DIM inhibits the CpG methylation of the first 5 CpGs on Nrf2 promoter, enhances the transcription of Nrf2 and Nrf2-target genes in the TRAMP prostate tissues. Moreover, DIM induced the protein expression of Nrf2 and an Nrf2-targeted gene NQO1 in the dorso-lateral prostate tissues (**Fig. 5B**), as compared to the prostate tumor samples from the untreated control TRAMP mice that were undetectable, which is consistent with our previous reported findings (131-135).

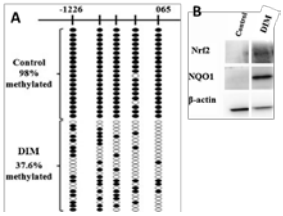


***Fig. 5.*** **(A) DIM demethylated Nrf2 promoter region in TRAMP prostate tissue. (B) Protein expression of Nrf2 and NQO1 in TRAMP prostate tissue.**

***C.1.2. Triterpenoids UA and BA active Nrf2-ARE pathway in HepG2-C8 cells and inhibit inflammatory responses in RAW cells.*** BA and UA have been reported to possess anti-inflammatory effects (136, 137). Fig. 6 shows that BA and UA (5-20 μM) induced Nrf2-ARE-luciferase activity in HepG2-C8 cells after 24 h. In addition, BA and UA in RAW 264.7 macrophage cells showed a dose-dependent inhibition in the expression of COX-2, iNOS, IL-1β, IL-6, and TNF-α, after pre-treating the cells with BA/UA for 1 h followed by 23 h of co-treatment of BA/UA with LPS (100 ng/mL) (**Fig. 7**). UA exhibited better reductive effect than BA.

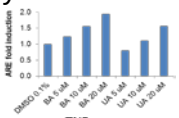


***Fig. 6.*** **UA and BA induce ARE-luciferase activity in HepG2-C8 cells.** The luciferase activity (ARE fold induction) was expressed as the fold induction over the vehicle treated cells after 24 hr treatment.

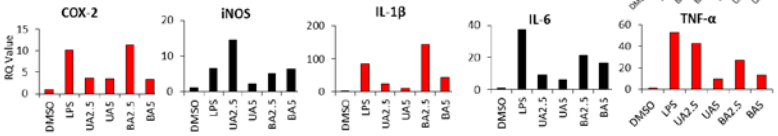


***Fig. 7.*** **UA and BA show anti-inflammatory effect in LPS induced murine RAW 264.7 cells.** The concentrations of UA and BA were used at 2.5 and 5 μM for pretreatment and then co-treatment with100 ng/mL of LPS.

***C.1.3. Triterpenoid UA epigenetically regulates the DNA methylation and the expression of HDACs and DNMT1 in vitro LNCaP cells.*** Previous reports show that BA possesses epigenetics modifying potential (120, 121). To test the potential epigenetic mechanisms of BA and UA, LNCaP cells were treated for 3 days with 2.5 μM of UA or BA and BGS showed that CpG hypermethylation of Neurog1 [a well-known positive control for epigenetic CpG modification (138)] in UA treated samples decreased by 18%, 5-aza (2.5 μM)+TSA (500 nM) by 15% compared to DMSO control treated (**Fig. 8A**). [We have recently reported that Neurog1 was epigenetically modified by curcumin in LNCaP cells (139); supported by NCCAM R01AT007065]. Furthermore, UA decreased the protein expression of DNMT1 and HDACs (HDAC2, HDAC3 and HDAC6), and BA decreased HDAC3 and HDAC4 when LNCaP cells were treated with BA and UA for 3 days (**Fig. 8B**).

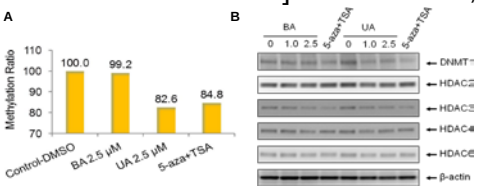


***Fig. 8.*** **(A) CpG demethylation effect of UA and BA on Neurog1 in LNCaP cells; (B) Effect of BA and UA on DNMT and HDACs protein expression.**

***C.1.4. Triterpenoids UA/BA and DIM and 5-aza+TSA induce Nrf2 and other anti-oxidant genes in mouse Pten-CaP2 cells and anti-inflammatory genes in LNCaP cells.*** Pten-CaP2 cells, which were derived from mouse prostate tumor of homozygous deletion of Pten allele (140), were treated with DIM, BA, UA, and 5-aza (2.5 μM)+TSA (500 nM) for 5 days. **Fig. 9** shows that DIM, BA, UA, and 5-aza+TSA induced mRNA expression of Nrf2 and Nrf2- target genes NQO1and HO-1 by qPCR analysis.

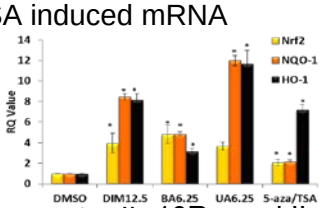


***Fig. 9.*** **Effect of BA, UA and DIM on the mRNA expressions of Nrf2-mediated antioxidant stress genes in Pten-CaP2 cells.** RNAs were extracted after 5 days of treatment from three independent experiments. Data are expressed as mean ± SD (normalized with β-actin and compared with control value). *p<0.05.

**Fig. 10** shows that in LNCaP cells, BA and UA induced the anti-inflammatory cytokine receptor IL-10Rα and IL-10Rβ after 5 days of treatment. Since 5-aza+TSA is a well-known epigenetic modifier (138), these results

suggest that UA and BA could also be considered to have epigenetically modifying potential on these genes, and further studies will be performed in Aim 3.

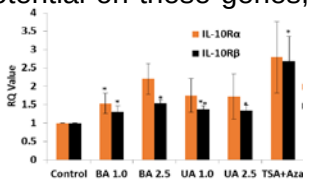


***Fig. 10.*** **Effect of UA and BA on the mRNA expressions of IL-10Rα and IL-10Rβ in LNCaP cells.** RNAs were extracted after 5 days of treatment from three independent experiments. Data are expressed as mean ± SD (normalized with β-actin and compared with control value). **p*<0.05.

***C.1.5. Epigenomics analysis of TRAMP prostate tissues and LNCaP-shSETD7 cells using MeDIP-seq:*** DNA was extracted from the prostate tissues of TRAMP and WT C57BL/6 mice at age of 24 weeks (pooled of 3 animals each),LNCaP-shSETD7 cells [LNCaP cells infected with lentivirus shRNA of SETD7, an epigenetic modifier histone-lysine *N*-methyltransferase (141), and stable clone was selected] and control LNCaP cells. MeDIP-seq (MeDIP-next generation sequencing) was performed at Otogenetics (Norcross, GA) (142, 143). About 14 million and 20 million pair-end reads were obtained using Illumina HiSeq2000 sequencer. Raw sequences were mapped and differentially methylated regions were identified using Bowtie, Tophat and Cufflinks programs in the Tuxedo Suite performed by Dr. Hart, a co-investigator in the Analytical Core (144). Genome annotation was performed using a Bioconductor package ChIPpeakAnno (145). Functional and pathway analyses were conducted using Ingenuity Pathway Analysis (IPA). Inflammation and oxidative stress related canonical pathways that are associated with CpG methylation changes are shown in **Fig. 11** and summarized in **Table 1**. In TRAMP prostate tumor, several signaling pathways of inflammatory and oxidative stress including IL-15, TGF-β, LPS-stimulated MAPK, PTEN, and Nrf2-mediated oxidative stress response are CpG hypermethylated, as compared to the WT C57/BL6 prostate tissue at 24 weeks. Similarly in LNCaP-shRNA-SETD7 cells, several inflammatory and oxidative stress pathways including IL-15, IL-16, IL-17A, IL-8, and NOS/ROS signaling are CpG hypermethylated, as compared to the control LNCaP cells (**Table 1**). These results suggest that the epigenomics of inflammatory and oxidative stress pathways are epigenetically modified in TRAMP prostate tumor as well as in shSETD7. The questions regarding the interactions of these inflammatory/oxidative stress epigenome and inflammation-mediated diseases as well as how CAM products triterpenoids and DIM will modify the epigenome resulting in inhibition of inflammation-mediated PCa will be the major goals of this Project.

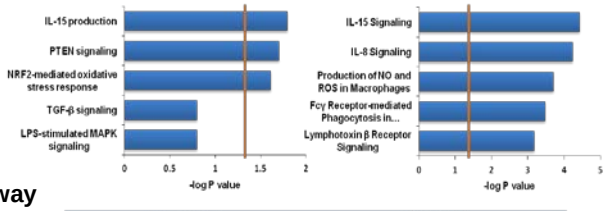


***Fig. 11.*** **Epigenomic analysis by MeDIP-seq followed by Ingenuity pathway analysis of TRAMP prostate tumors and LNCaP-shSETD7 cells show the top signaling pathways that were found to be associated with DNA methylation changes.** The blue bars represent *p*-value of each pathway, and the red lines indicate the threshold of *p*-value 0.05 (-log p-value =1.3).

| TRAMP Prostate | | LNCaP shSetd7 | |
|---|---|---|---|
| Signaling Pathways | Genes under this pathway | Signaling Pathways | Genes under this pathway |
| IL-15 Production | L6, JAK1, PRKCZ | IL-15 signaling | AKT2/3, IL4, IL17A, JAK2/3, NFκB2, PIK3C3 |
| PTEN signaling | CASP9, FOXO6, PIK3CD, PIK3R3,PRKCZ | IL-8 signaling | AKT2/3, BAX, BCL2L1, IL9, VEGFC, NOX1/3, |
| Nrf2 mediated oxidative stress response | AKR1A1, AKR7A2, SOD3, TXN, PRDX1, PRKCZ, JUN, MAP3K7 | Production of NO and ROS in macrophages | AKT2/3, FOS, IKBKB, JAK2/3, NFκB2, NFκBIA, PIK3C3/2A/2B/2G, TNFRSF1A |
| Wnt/GSK-3β signaling | WNT10A, IFNB1, WNT4, WNT6, INFK | FCγ mediated phagocytosis | ACTC1, AKT2/3, MAPK1, PIK3CG |
| | | Lymphotoxin β receptor signaling | MAPK1, NFKB2, NFKBIA, PIK3C3, TNFSF14 |

***Table 1*** **The genes of the signaling pathways that are potentially regulated and will be used as candidate genes.**

***C.1.6. DIM and triterpenoids UA and BA down-regulated onco-microRNA in LNCaP cells:*** *DIM, BA and UA have been reported to have anti-cancer activity by triggering various miRNA-mediated pathways (116-119, 121, 127). DIM (10 μM), BA (5.0 μM) and UA (2.5 μM) decreased the expression of onco-miRNAs, miR18 and miR106, in LNCaP cells after 24 hr treatment (**Fig. 12**).*

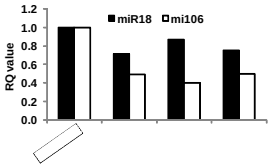


***Fig. 12.*** ***DIM, UA and BA decrease expressions of miR18 and mi106 in LNCaP cells.*** *miRNAs were extracted after 24 hr of treatment and normalized with U6 and compared with control value.*

## C.2. Detailed Research Design: Methodology and Analyses

**C.2.1. Aim 1: To investigate the alterations of inflammatory and prostatic epigenetics in Pb-Cre/Pten(flox/flox) mice, PhIP induced CYP1A humanized mice and TRAMP mice, to evaluate the preventive efficacy of triterpenoid UA and I3C, and how these CAM products target the epigenomic regulation of anti-inflammation/oxidative stress genes.**

***Rationale:*** Many studies have shown that prostatic inflammation and oxidative stress contribute to human prostate carcinogenesis (11, 12, 14-17). Chronic inflammation occurs due to macrophages, lymphocytes and plasma cells infiltrating damaged tissue. All of these leukocytes can release potentially harmful products such as ROS, cytokines, and other mediators, which contribute to the onset of carcinogenesis (146). The PTEN tumor suppressor gene is one of the most frequently mutated/deleted genes in various human cancers (147). PTEN deletions and/or mutations are found in 30% of primary PCa and 63% of metastatic prostate tissue samples, placing PTEN mutation among the most common genetic alterations reported in human PCa. The mouse model using Pb-Cre promoter and Pten-flox has been commonly used to examine the role of Pten deletion and prostate tumorigenesis (148). With prostate specific Pten null mice, at week 10 – low grade (lg)

PIN, at week 12 – high grade PIN, and adenocarcinoma develops starting week 12 to week 20 (at week 20, 100% mice have adenocarcinoma (11, 26, 27). Prostate specific Pten-null mice showed increased expression of CXCL8/IL-8, a pro-inflammatory chemokine promoting tumorigenesis (25). Our Preliminary Studies in **Fig. 13**, shows that there are enhanced inflammatory infiltrates cells in the prostate specific Pten null mouse. Furthermore, many cancer preventive agents including anti-inflammatory PUFAs (149-151), resveratrol (152), and SFN (153) inhibited PCa of PTEN null mice.

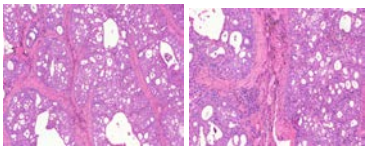

***Fig. 13.*** **Prostate tissue from 18 weeks old Pten prostate specific KO stained with H&E.** *Left (20X); Right (40X): inflammation are seen infiltrating the muscle in the right panel.*

In addition, it has been reported that the majority of epigenetic CpG methylation changes detected in cancer cells are acquired during neoplastic development and therefore specific to cancer (154), and that epigenetic changes arise earlier than genetic defects. As we have reported, the expression of Nrf2 and Nrf2-target genes are suppressed in prostate tumors of the TRAMP mice (128, 132, 155) and that Nrf2 gene is epigenetically silenced via CpG methylation during the development and progression of prostate tumorigenesis in TRAMP mice (128). Furthermore, we have recently shown that when TRAMP mice were fed with dietary broccoli sprouts (155), γ-TmT (132), and CUR+/-PEITC (131), which inhibited tumorigenesis, re-expression of Nrf2 and Nrf2-target genes were observed. From our Preliminary Studies, DIM induces HepG2-C8-ARE-luc at 25 and 75 μM in 24 h (**Fig. 1**); DIM suppresses protein expression of DNMT and HDACs in TRAMP C1 cells with much lower doses (2.5 and 5 μM) of DIM for 5 days (**Fig. 2**); DIM demethylates CpGs of promoter of Nrf2 gene in TRAMP C1 cells (**Fig. 3**) and *in vivo* TRAMP tumors and re-expressed Nrf2 and Nrf2 target genes NQO1 (128) (**Fig. 4**); UA would epigenetically demethylate CpG of the promoter of Neurog1 (**Fig. 8A**) and decrease protein expression of DNMT1, HDAC3 and HDAC6 (**Fig. 8B**) similar to 5-aza+TSA; UA enhance Nrf2, Nrf2 target genes NQO1 and HO-1 genes in Pten-CaP2 cells (**Fig. 9**), and anti-inflammatory IL-10Rα & IL-10Rβ in LNCaP cells (**Fig. 10**) at low 2.5 μM doses in 5 days, similar to positive control epigenetic modifier 5-aza+TSA; and using MEDIP-seq NGS, several signaling pathways of inflammatory and Nrf2-mediated oxidative stress are CpG hypermethylated in TRAMP prostate tumor and in LNCaP-shRNA-SETD7 cells (**Fig. 11 & Table 1**). In this Aim, we will investigate the epigenome/epigenetic alterations (inflammation genes and anti-oxidative stress genes) at different stages of Pten-null, PhiP-induced inflammation/high-grade PIN (HGP) in CYP1A-humanized mouse and TRAMP mouse prostate tissues and the preventive effects of UA and I3C via epigenetic modifications. The results of the 3 mouse models, including epigenome/epigenetic alterations of anti-inflammation/oxidative stress genes and preventive efficacy of UA and I3C, will be compared and contrasted.

***1a. Studies on anti-inflammatory efficacy and effects of UA and I3C on the epigenetic alterations at different stages of Pten-null mice.*** The generation of PTEN(f/f) breeding has been ongoing in our laboratory and F1 mice were generated by crossing male Pb-Cre4 mice from NCI (01XF5) with female Pten(flox/flox) from Jax lab. The mice will be genotyped and only the male mice with cre-carriers and homozygous Pten$^{flox/flox}$ will be used for the treatment groups. All mice will be housed in our animal facility in accordance with protocol approved by the Rutgers University Institutional Animal Care and Use Committee. All mice will be maintained under standard 12-h light/12-h dark cycles with water and diet provided *ad libitum* unless otherwise specified. *In Preliminary Study, we found that significantly lower mRNA levels of Nrf2 and NQO1 as well as significantly higher mRNA levels of COX2, iNOS and IL6 in the prostate tissues of PTEN null mice as compared to control Pb-cre/Pten(f/f) mice (**Fig. 14A & 14B**). Global methylation status shows that DNA hypermethylation is significantly decreased in ventral & lateral prostate (VLP) of PTEN null as compared to control mice (**Fig. 14C**).*

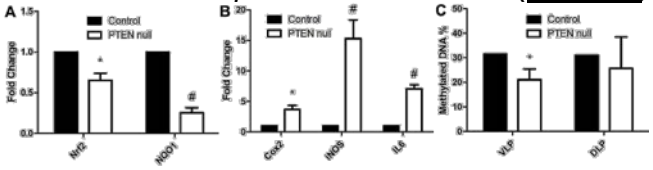


***Fig. 14.*** ***Differences between control PTEN(f/f) and PTEN null mice.*** *(A) Relative mRNA expression of Nrf2 and NQO1; (B) relative mRNA expression of COX2, iNOS and IL6; (C) the percentage of global DNA methylation. Data are expressed as mean ± SD (n=3; normalized with β-actin and compared with control value). *p<0.05; # p<0.01.*

To further the study, the treatment groups (n=20 per group per time point) of Pb-cre/Pten(f/f) mice will include a control group, UA (≥90% purity, Sigma-Aldrich U6753) 0.1% w/w kg diet based on published data (156), and I3C (≥96% purity, Sigma-Aldrich I7256) 1% w/w kg diet. From Preliminary Study (**Fig. 5**) and published results, the *in vivo* efficacy of I3C and DIM are similar (115, 135). The mice will be fed with the diet *ad libitum* and they will be sacrificed at 10, 20 and 30 weeks. The general health of the mice will be monitored throughout the experimental period. The body weights of the mice will be recorded weekly. At the end of each time point the mice will be euthanized and blood, prostate will be collected, dissected and weighed. Prostate tissues (n=11 out of 20 per group) will be fixed in 10% buffered formalin for pathology study (described below in *1d*), and the remaining nine will be snap-frozen in liquid nitrogen and stored in -80°C for epigenomics and biomarkers analysis (n=6; described below in *1e*) and chemical analysis (n=3; *1f*). We will proceed first with UA based in

part on published literature (126) and our Preliminary Study on epigenetics effects (**Fig. 10**). DIM and other triterpenoids such as BA and CDDO will be considered (115, 120, 157).

***1b. Studies on anti-inflammatory efficacy and effects of UA and I3C on the epigenetic alterations at different stages of PhiP-induced inflammation/HGP in CYP1A-humanized mice.*** In CYP1A-humanized mice, PhIP-induced inflammation/HGP in the prostate coupled with increased inflammatory markers such as 8-oxo-dG, nitrotyrosine, COX-2, p-AKT, and Pten (**Fig. 15**) has been reported (37). In this project, male *Cyp1a2/Cyp1a1*$^{tm2Dwn}$ Tg(*CYP1A1,CYP1A2*)1Dwn/DwnJ C57BL/6J mice (*Cyp1a1,Cyp1a2*) were purchased from Jackson Laboratories and used as founders to establish homozygous breeding colonies in our animal facility(37). All mice will be housed in our animal facility in accordance with protocol approved by the Rutgers University, and maintained under standard 12-h light/12-h dark cycles with water and diet provided *ad libitum* unless otherwise specified. The treatment groups (n=20 per group per time point) of hCYP1A mice will include control group, PhIP group, PhIP+UA (0.1% w/w), and PhIP+I3C (1% w/w) similar to *1a* above and UA and I3C will be given in AIN-93M diet. hCYP1A mice and wild-type mice will be treated with a single dose of PhIP (200 mg/kg body weight) by oral gavage in distilled water at 6 weeks of age (37). All hCYP1A mice will be included as vehicle (distilled water) control. Mice will be maintained on an AIN-93M diet. Body weight, food intake and monitoring for general health will be taken weekly. To examine the changes of the epigenome (focusing on inflammation and oxidative stress) during the progression of inflammation/PIN lesions, PhIP-treated and control mice will be euthanized by $CO_2$ asphyxiation at 10, 20, 30 or 40 weeks after PhIP treatment (37). Prostate will be dissected and weighed. Prostate tissues (n=11/20 per group) will be fixed in 10% buffered formalin and the remaining nine will be snap-frozen in liquid nitrogen and stored in -80°C for biomarkers and epigenomics (n=6; *1e*) and chemical analyses (n=3; *1f*). After fixing in formalin, the entire prostate lobes (anterior, dorso-lateral complex and ventral) will be embedded in paraffin wax and histopathologic analyses of the entire prostate tissues will conducted on multiple serially sectioned at 4 µm sections per block as described below in *1d* as previously (37, 115, 133, 158). The frozen prostate will be grinded in liquid nitrogen and split into three parts for extraction of DNA and RNA and measurement of compound concentration. We will proceed first with UA based in part on published literature (126) and our Preliminary Study on epigenetics effects (**Fig. 11**). We will consider DIM and other triterpenoids such as BA and CDDO (115, 120, 157), and the rat PhIP model as we proceed.

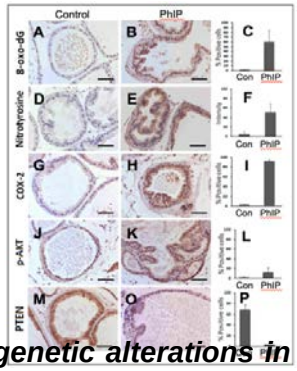


***Fig. 15.*** **PhIP treatment induced the elevation of 8-oxo-dG, nitrotyrosine, COX2 and pAKT expressions and the loss of PTEN expression in the dorso-lateral prostate (DLP) of hCYP1A mice at week 40.** A, D, G, J & M – from control mice (received no PhIP); B, E, H, K & O – from mice received PhIP); C, F, I, L & P – quantification of the levels (n=5 or 4).

***1c. Studies on anti-inflammatory efficacy and effects of UA and I3C on the epigenetic alterations in* TRAMP *mice.*** We will examine the epigenetic modifications and prevention effects of UA and I3C during prostate tumor development and progression in TRAMP mice at 16 and 24 weeks. The dosing levels of I3C and UA, and the AIM-93M diet will be the same as above in *1a & 1b*. The mice will be weighed and monitored weekly. At the end of the experiment, the mice will be sacrificed and the genitourinary (GU) apparatus consisting of the seminal vesicles, prostate and bladder will be weighed, prostate samples will be proportioned for IHC (n=11), biomarkers/epigenomics (n=6) and bioanalytical (n=3), as described in *1a & 1b* above. The incidence of mice with palpable prostate tumor will also be recorded and confirmed upon autopsy. The distant metastasis such as lymph modes will be examined as we have published (131, 132, 158). To corroborate the tumor efficacy results, we will examine the epigenetic modifications after UA and I3C treatments.

***1d. Pathological and histopathological analyses.*** At each stage of sampling periods (from Aims 1a, b & c), the mice will be sacrificed by $CO_2$ asphyxiation, prostate (n=11) will be collected and weighed. The prostate tissue will be processed with hematoxylin and eosin (H&E) staining for pathological and histopathological analyses, performed in a blinded fashion by a designated pathologist; Dr. Li, a Pathologist in this application (please see attached letter). Cell apoptosis and proliferation will be examined using tunnel assay and PCNA staining. At least 5 slides from each animal will be examined for proliferation and apoptosis profiles.

***1e. Epigenomics/Bioinformatic and biomarkers analyses: (1) Epigenomics/bioinformatic analysis:*** The DNA samples from the above experiments will be used for the DNA Methylation Reduced Representation Bisulfite Sequencing (RRBS) - gene silencing, and enhanced chromatin H3K27ac-ChIP-seq, performed with the Analytical Core's Next-generation Sequencing (NGS) section (attached in Appendix), under Dr. Brooks, Associate Professor, Genetics and Technology Director & Chief Operating Officer, RUCDR. The details are described in Analytical Core and briefly summarize below. ***(2) Genome-wide promoter DNA methylation status of inflammation and oxidative stress related genes:*** Prostate tissues (6 pooled samples for each

group) from *in vivo* studies above (***1a, 1b & 1c***) will be harvested for extracting DNA/RNA simultaneously using Allprep-DNA/RNA-mini kit (Qiagen). DNA samples will be used for the enhanced form of RRBS (159), which is a DNA methylation research methodology focusing on genome-wide CpG-rich regions and promoters. We will perform bisulfite conversion first in the lab and the sequencing will be done by our Analytical Core (attached in Appendix) using the Illumina HiSeq2500 (as backup, BGI also provides this service). RRBS, which has single base resolution, fewer data waste, and lower cost, would provide us the data that can be extended for further gene associated research (e.g. cell signaling, structure, etc.). The results of RRBS will be compared with transcriptome RNA-seq profiles (below) to identify the true meaningful epigenetic changes and to identify the impacted pathways by our Analytical Core (attached in Appendix). ***(3) RNA-Seq:*** RNA samples will be used for obtaining transcriptome by quantification RNA-seq in collaboration with Dr. Hart (Analytical Core), as described previously (160, 161). The results will be used to correlate epigenetic changes and alteration on gene expression. For genes associated with the increase of DNA hypermethylation or suppressed enhancer chromatin (via reduced H3K27ac region; see description below), we expect down-regulated expression. For genes associated increase of DNA hypomethylation or activated enhancers (via increased H3K27ac or H3K4me1 region), epigenetic change may not result in the up-regulation of expression but only the ones that lead to expression changes are significant for further evaluation. In addition, pathway analysis of this data will provide information for understanding the consequence of epigenome changes. Transcriptome profiles will offer us the data of the gene expression that are not controlled by epigenetic mechanism for future research. Moreover, we will use bioinformatics approach to compare the DNA methylation data with histone modification data. ***(4) Histone modification by MNase-H3K27ac or MNase-H3K4me1 CHIP-seq:*** The experimental procedure for MNase-H3K27ac CHIP-seq will be performed in collaboration with Dr. Verzi (Analytical Core), as previously described (162). In brief, we will perform the MNase digestion and H3K27ac or H3K4me1 CHIP. Sequence of ChIP products will be done by our Analytical Core with the Illumina HiSeq2500 (as backup, BGI also provides this service). Single-end sequence data will be filtered and aligned by our Analytical Core, and H3K27ac- or H3K4me1-modified nucleosomes will be called using NPS (Nucleosome Positioning from Sequencing) (163). NSD (Nucleosome Stabilization–Destabilization) analysis will be performed to identify highly dynamic chromatin regions between conditions (164). Dynamic chromatin regions are expected to correlate with transcriptome changes (tested by comparing the frequency that dynamic chromatin occurs nearby regulated genes versus non-regulated genes using Fisher's exact test and a 10kb distance cutoff as we have done previously (162). Nearby genes will be identified using Cistrome's Peak2Gene (165) and analyzed using pathway analysis. Finally, dynamic regions will be explored for transcription factor binding using SeqPos (165) to identify transcription factors mediating the epigenomic changes associated with inflammation/oxidative stress. **(5) Regulatory RNA:** The major classes of regulatory RNA include microRNAs (miRNAs, usually 18-21 nt long; most function to reduce translation or stability of target mRNAs) and long noncoding RNAs (lincRNAs, normally spliced but lacking recognizable open reading frames for translation). We will study cell culture samples for changes in global miRNA populations using the Affymetrix GeneChip miRNA 4.0, which detects 2,578 mature human miRNAs (160, 166-170). The arrays will be run by RUCDR (Analytical Core), which has a large and robust Affymetrix program with robotic probe preparation pipelines. Follow-up experiments will use qPCR or similar methods. For lincRNAs, RNAseq datasets will be aligned to genome using transcript maps obtained from Dr. Hart's (Analytical Core) collaboration with the Rinn Lab (169). ***(6) Bioinformatic analysis:*** Bioinformatic analysis will be done in close collaboration with Dr. Hart (Analytical Core's Bioinformatics Co-Principle Investigator) and the methylome DNA sequencing reads and results from MNase-H3K27ac/H3K4me1 CHIP-seq will be analyzed *as we have published recently (171)* and described in details in the Analytical Core's Bioinformatics. All the changes of methylation related genes will be further processed using Ingenuity software and other bioinformatics approaches to investigate how these epigenetic modulations by UA and I3C are associated with its anti-inflammatory/oxidative stress activities. At present, we cannot predict what this data may offer. We speculate that we may see the correlation in most of genes but we may also see some that are not associated with each other. Nevertheless, this information will help us to understand what kind of epigenome changes can be reversed by I3C and UA. The expression of aberrantly silenced genes will be validated using qPCR and Western blotting. For these validation studies, we will first focus on inflammatory markers such as COX-2, iNOS, IL-6, IL-1b, TNF-α, and anti-oxidative-stress markers including Nrf2 and Nrf2-target genes GSTs, NQO-1 and UGT1A1. Other inflammatory/oxidative stress genes will be performed as we proceed. Moreover, we will also determine mRNA and protein expression of DNMTs and HADCs in the mouse prostate tissues to understand the possible preventive mechanism of UA and I3C via epigenetics modifications.

***1f. Determine blood and tissue levels of I3C and UA and their metabolites and correlates to the epigenetic effects:*** In order to relate the CAM products and metabolites concentrations of I3C and UA *to* the

epigenetic modifying effects, we will determine the concentration of testing compounds and their metabolites in the blood as well as in the prostate, using HPLC and/or LC-MS as we have published previously (155, 158, 172-174). These assays are routinely performed in our laboratory and in our Analytical Core (attached in Appendix), under Dr. Brian Buckley, Adjunct Associate Professor, Rutgers School of Public Health and Executive Director Laboratories, EOHSI. We have developed and validated a UPLC-MS method to measure I3C, DIM, and their metabolites simultaneously. Quantification of UA will be performed using a published HPLC-MS method (175).

***1g. In vivo pharmacokinetics (PK) and pharmacodynamics (PD) studies of I3C/DIM and UA after intravenous and oral administrations:*** A single i.v. dose of 5 mg/kg of UA or I3C/DIM (in PBS containing 0.5% Tween 80) and an oral dose of 75 mg/kg (in olive oil) for each compound will be administered to four groups each comprising eight male Sprague-Dawley rats. Another two groups of rats will be dosed with vehicle only. At 5 (i.v. dose only), 15, 30, and 60 minutes and 2, 4, 8, 12, 18, and 24 hours following I3C/DIM or UA administration, 0.3 mL of blood from each rat will be drawn. Plasma and mononuclear cells will be obtained as described (176). The plasma concentration of I3C and UA will be determined using UPLC-MS or HPLC-MS methods (*Aim 1f*). Relative mRNA expression of Nrf2-target genes (NQO1, UGT1A1, UGT1A6, GSTM1, GSTT1, HO-1, and GPx-1) and inflammatory genes (COX2 and iNOS) in blood mononuclear cells will be used as PD markers determined by qPCR (other genes will be considered). The PK parameters for compounds and its metabolites including half-life, clearance (CL), and volume of distribution (Vss) will be calculated using WinNonLin and ADAPT5 softwares, as performed previously (155, 172, 174, 176). The plasma concentration profiles will be compared to concentration measured in Pten, PhIP and TRAMP mice (*Aims 1a, b & c*). We have performed a preliminary PK-PD rat study to characterize the relationship of plasma DIM concentration and mRNA level of Nrf2-mediated NQO1 in blood mononuclear cells. mRNA level of NQO1 was induced after DIM administration (10 mg/kg, i.v.) and then returned to the basal level when DIM was eliminated from the body (**Fig. 16**).

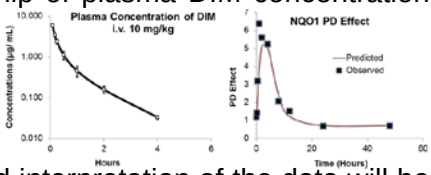


***Fig. 16.*** **PK-PD profiles of DIM (10 mg/kg i.v.) in the rats.**

***1h. Statistical analysis and sample size:*** Data analysis, study design and interpretation of the data will be performed in coordination with the Analytical Core's Statistics section under Dr. Shih, Professor, Department of Biostatistics, Rutgers School of Public Health, a collaborator on this application and a co-Investigator on two currently funded R01 grants. One way ANOVA model will be used to test whether the treatment effects on compounds are significantly different from the control AIN93M group or not. We will use alpha = 0.05 as the level of significance for hypothesis testing. A sample size of 20 mice per group will give about 80% power for detecting treatment effects similar to what we have published previously (177, 178).

***1i. Benchmarks for success, potential problems and alternative strategies anticipated for Aim1:*** We do not anticipate any problem with the technical aspect of our studies. We anticipate that UA and I3C will be effective in blocking inflammation in Pten-null, PhiP-induced inflammation/HGP in CYP1A-humanized mice and TRAMP mice. We anticipate to discover new and novel epigenetic alterations associated with the prostate inflammation in different mouse models. UA and I3C may exert its preventive effects of inflammation/oxidative stress and PCa through epigenetically restoring or preventing the aberrant epigenetic alterations associated with inflammation/oxidative stress in our models. Although the *in vitro* activity of DIM is more potent than that of I3C in various cell lines, I3C and DIM exhibit very similar *in vivo* efficacy (115, 135). We may consider alternative prostate inflammatory models such as prostatitis model using non-bacterial prostate inflammation (179, 180) or transgenic mouse model induced by injection of immune cells (CD8+ T cells) (181). The role of aryl hydrocarbon receptor (AhR) and androgen receptor (AR), which are associated with I3C and PCa, in immunomodulation will be considered (182-184). *It was reported that prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of PCa with a basal cell origin in a Pten KO model (185). Our Preliminary Study found that E. coli-induced prostatic inflammation in our Pten null mice (data not shown), hence this experimental model will be considered as another alternative strategy. Additionally, we will conduct pilot study to detect novel modified nucleotides (e.g., 5hmC, 5f-C, 5ac-C) as well as Tet family of dioxygenases as described in details under Analytical Core Aim 3* (attached in Appendix).

**C.2.2. Aim 2: To investigate the inflammation/apoptotic epigenome profiles in human VCaP PCa cells in NCr(-/-) nude mice, and to investigate the effect of UA and I3C on the anti-inflammatory/pro-apoptotic epigenome pathways.**

***Rationale*** In our Preliminary Studies, we found that UA and I3C/DIM are cytotoxic in PCa cells, VCaP, LNCaP, TRAMP C1, and Pten-CaP cell lines (data now shown). We have also shown that I3C/DIM and UA suppress LPS-induced inflammation in RAW 264.7 macrophage cells (**Fig. 7**), and UA epigenetically demethylated the

promoter of Neurog1 (**Fig. 8A**) that would contribute to apoptotic cell death (139). Additionally, inhibition of inflammatory pathways such as NF-κB would cause cell apoptosis (186, 187). In this Aim, we will evaluate the efficacy of UA and I3C in inhibiting VCaP prostate tumor growth in NCr(-/-) nude mice. We will investigate their effects on anti-inflammation and pro-apoptotic epigenomic modifications in VCaP tumors. VCaP cells are androgen sensitive human PCa cells derived from a vertebral bone metastasis in a patient (188). Other human PCa cells such as LNCaP or PC3 (189, 190) will be considered.

***2a. Evaluation of the efficacy of UA and I3C inhibiting xenografted VCaP tumors in NCr(-/-) nude mice.*** Male NCr(-/-) mice of 6-7 weeks old will be housed in sterile filter-capped microisolator cages in the Animal Facility at Rutgers and provided with sterilized food and water. After subcutaneous injection of VCaP-luc cells, NCr(-/-) NUDE mice will be randomly assigned to three groups including control group, UA and I3C. UA or I3C will be given in AIN-93M diet for oral administration (*Aim 1a*), for 8 weeks. Each group will contain 20 mice. During the study, tumor size in VCaP-luc xenografted mice will be monitored weekly using the IVIS imaging system (**Fig. 17**). Body weight will be measured once every third day. At the end of study, the mice will be sacrificed and tumors will be excised, weighed, and analyzed as described above in *Aims 1a, b & c*. Blood samples will be collected for the determination of plasma concentrations of I3C/DIM or UA and their metabolites, as described in *Aim 1f.*

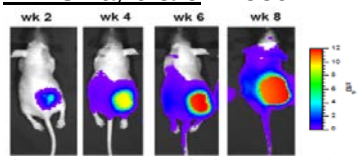


***Fig. 17.*** **Growth of VCaP tumor in NCr(-/-) nude mice after subcutaneous implantation.** Luminescent signal was measured as the photon flux (ph/s).

***2b. Pathological and histopathological analyses.*** Tumor tissue (n=11) will be processed for IHC for pathological and histopathological analyses. Apoptosis, proliferation and inflammation will be examined using tunnel assay, PCNA, COX-2, and iNOS staining (other markers will be considered). At least 5 slides from each animal will be examined for each marker as described above in *Aim 1d*.

***2c. Bioinformatic analyses:*** DNA and RNA will be collected from VCaP tumors in the control and treated groups (n=6 per group). Similar to *Aims 1a, b & c* above, DNA or RNA from three mice will be pooled and two biological replicates in each group for RRBS, H3K27ac/H3K4me1-ChIP-seq, RNA-seq, and *regulatory RNA* studies as described in *Aim 1e*. We will identify regions with differential DNA methylation and H3K27ac/H3K4me1, and differential transcripts levels by UA and I3C. We will determine the correlation between DNA methylation and H3K27ac/H3K4me1, transcripts expression and DNA methylation, and transcripts expression and H3K27ac/H3K4me1. The expression of aberrantly silenced genes will be validated by qPCR and Western blotting. We will first focusing on inflammatory markers such as COX-2, iNOS, IL-6, IL-1b, TNF-α, and pro-apoptotic genes such as ASC (apoptosis-associated Speck-like protein containing a CARD), BCL2 (B cell lymphoma 2) and DAPK (death-associated kinase), which have been shown to be silenced due to DNA methylation in PCa (191, 192). We will also determine mRNA and protein expression of DNMTs and HADCs in the mouse prostate tissues to understand the possible preventive mechanism of UA and I3C/DIM via epigenetics modifications.

***2d. Determine levels of testing compounds and their metabolites in blood and tissues:*** The concentrations of I3C/DIM and UA and their metabolites will be measured in blood, tumor and prostate (n=3) as described above in *Aim 1f*.

***2e. Statistical analysis and sample size:*** Data analysis, study design and data interpretation will be performed in coordination with the Analytical Core's Statistics under Dr. Shih as described above in *Aim 1h*.

***2f. Benchmarks for success, potential problems and alternative strategies for Aim 2:*** We anticipate I3C and UA will inhibit the growth of VCaP xenograft tumors in NCr(-/-) nude mice, associated with suppressed inflammation/proliferation and enhanced apoptosis. We expect to identify epigenetic alterations resulting from UA and I3C treatments. We do not expect any technical difficulties. Alternative human PCa LNCaP and/or PC3 xenografts as well as other epigenetically modifiable inflammatory and apoptotic genes and other triterpenoids or DIM will be considered. The results of *Aim 2* will be compared to the results of *Aim 1* above.

**C.2.3. Aim 3: To elucidate *in vitro* molecular epigenetic mechanisms in the regulation of the inflammatory/oxidative stress and pro-apoptotic genes obtained from *in vivo* Aims 1 and 2 above by *UA and DIM*, in mouse TRAMP C1, mouse Pten-CaP2, and human VCaP and LNCaP PCa cell lines.**

***Rationale*** We hypothesize that the *in vivo* inflammation preventive mechanisms of CAM products such as I3C/DIM and triterpenoids are due to their potential epigenetic modifications leading to anti-inflammatory/anti-oxidative stress signaling pathways in Pten-null, in PhiP-induced inflammation/HGP in CYP1A-humanized mouse and in TRAMP mice as well as anti-inflammatory/pro-apoptotic pathways in human VCaP PCa cells in NCr(-/-) nude mice. In this Aim we will investigate epigenetic mechanisms of CAM products-mediated anti-inflammation, anti-oxidative stress and pro-apoptotic genes *in vitro* cell lines which will complement and

provide mechanistic insights to our *in vivo* studies described above in Aims 1 & 2. We will test our hypothesis of UA- and I3C/DIM-induced epigenetic modifications in PCa cell lines: mouse TRAMP C1, mouse Pten mutation (Pten-CaP), and human VCaP and LNCaP cell lines. We will include normal RWPE-1, epithelial cells derived from the peripheral zone of a histologically normal adult human prostate transfected with a single copy of the human papilloma virus 18 (ATCC CRL-11609), as normal control. *Since DIM is more potent than I3C in many cell lines, they appear to have similar in vivo activities in our Preliminary studies (115, 135), DIM will be used throughout Aim 3, unless otherwise indicated.*

***3a. To examine whether UA and DIM can reverse the DNA methylation status of the promoters of inflammation, and anti-oxidative stress genes in TRAMP C1 and Pten-CaP cell lines in vitro:*** We will treat TRAMP C1 and Pten-CaP2 cells with different concentrations of UA (≥90% purity, Sigma-Aldrich U6753) and DIM (≥98% purity, Sigma-Aldrich D9568) for different durations as performed in our Preliminary Studies with DIM *(DIM, the active metabolite of I3C will be used in Aim 3, unless otherwise indicated)* and UA (**Fig. 2, 3, 4, 7, 8, 9 & 10**). Sodium-bisulfite conversion will be performed using the EZ DNA methylation kit (Zymo Research) as done previously (128, 130, 134, 139, 193). We will use ChIP-qPCR to examine the proteins that could be potentially associated with gene promoters in TRAMP C1 and Pten-CaP cells and whether DIM or UA affect the association of these proteins (MBD2; MeCP2:methylated DNA binding protein; H3K27me3: transcription repression; H3K4me2 & H3K27ac: transcription activation) leading to re-expression or suppression of inflammation and anti-oxidative stress genes as we have performed recently (128, 130, 134, 139, 193). In addition, those genes that are clinically relevant will be selected for further study. The correlation between DNA methylation and mRNA/protein expression levels of these genes will be examined using qPCR and Western blotting.

***3b. To examine whether UA and DIM reverse the DNA methylation status of the promoters of hyper/hypo-methylated genes in human PCa* VCaP and LNCaP cell lines *in vitro***: As shown in our Preliminary Results, DIM and UA demethylated and re-expressed Nrf2 *in vitro* TRAMP C1 cells (**Fig. 3**), *in vivo* TRAMP prostate tumor (**Fig. 4**), and Neurog1 in LNCaP cells (**Fig. 8A**). To investigate how human PCa cells can also be epigenetically modified by UA and DIM (same as *3a*), we will evaluate inflammation-related genes such as IL-10Rα, IL-10Rβ, IL1R1, IL4R, IL-1β, TLR2, 15-LOX,COX-2, and Foxp3, as well as pro-apoptotic genes such as ASC, BCL2, DAPK, and Neurog1 using bisulfite sequencing (Preliminary Results; **Fig. 8A**). We will clone the promoter region of these genes of interest into a luciferase reporter vector as described (128) to verify the effect of methylation on their transcriptional activity. In addition, we will use ChIP-qPCR to examine the proteins (MBD2, MeCP2: methylated DNA binding protein; H3K27me3: transcription repression; H3K4me2 & H3K27ac: transcription activation) that would be potentially associated with the promoter of the selected genes in VCaP and LNCaP cells and whether UA and DIM treatments would affect the association of these proteins leading to re-expression of these genes.

***3c. To investigate the effects of UA and DIM on the DNMTs and HDACs mRNA and protein expression.*** We will treat TRAMP C1, Pten-CaP2, VCaP, and LNCaP cells with UA and DIM for 3, 5 and 7 days at different concentrations (**Fig. 2, 3, 4, 8, 9, & 10**). mRNA and protein will be extracted from the cells for qPCR and Western blotting to detect the mRNA and protein expression levels of DNMT1, DNMT3a, DNMT3b and HDACs (e.g. HDACs 1, 2 and 3) (128, 130, 134, 139, 193).

***3d. To examine histone methylation/acetylation on the chromatin packaging regulation coupled to DNA methylation status changes by UA and DIM in TRAMP C1, Pten-CaP2, VCaP, and LNCaP cells*:** Our Preliminary Studies show that DIM inhibited DNMT1 and HDACs in TRAMP C1 cells (**Fig. 2**), demethylated Nrf2 (**Fig. 3**), re-expressed Nrf2 and Nrf2-target genes (**Fig. 4**) in TRAMP C1 cells and *in vivo* TRAMP prostate tumors (**Fig. 5**). UA demethylated Neurog1 in LNCaP cells (**Fig. 8A**) and inhibited DNMT1 and HDACs (**Fig. 8B**) [similar to curcumin (139)]. Furthermore, DIM and UA induced expression of Nrf2 and Nrf2-target genes (**Fig. 9**) in Pten-CaP2 cells [We have reported that Nrf2's CpG is hypermethylated in TRAMP C1 cells and in TRAMP prostate tumors (115, 128, 130, 134, 193, 194)], and UA induced IL-10Rα and IL-10Rβ in LNCaP cells (**Fig. 10**), similar to the positive control 5-aza+TSA. Lentivirus shRNA knock-down (KD) of histone-lysine *N*-methyltransferase SETD7 (shSETD7) in LNCaP cells modified CpG methylation of genes of various inflammatory and oxidative stress pathways (**Fig. 11 & Table 1**) [Currently we have generated 17 human and 15 mouse shRNA-KD of histone acetylases (HATs), HDACs, histone methyltransferases (HMTs) and histone lysine demethylases (KDMs) with many stable cell lines generated in LNCaP, TRAMP C1 and JB6 mouse epidermal cells]. Many reports have shown that CAM products including SFN/PEITC, polyphenols such as EGCG, curcumin, luteolin, and triterpenoids inhibit HDACs (96, 139, 195-198). From our unpublished works on methyltransferases screening, CAM product such as SFN inhibited the expression of multiple KDMs, including KDM1, KDM3a, KDM4a, KDM4b, KDM4c, KDM5a, and KDM5b but not KDM3b and KDM4d when treated with

2.5 μM SFN. From the stable LNCaP, TRAMP C1 and Pten-CaP2 cells expressing individual sh-HATs, sh-HDACs, sh-HMTs, and sh-KDMs, we will investigate whether each of the decreased expression of each histone modifier associates with the DNA methylation status alteration based on the BGS sequencing analysis as we had published previously (115, 128, 130, 134, 193, 194). Once the effect of specific histone modifiers on the DNA methylation determined, we will trace back to lysine modifications in histone 3 and histone 4, thereby establishing the link between the histone acetylation/methylation status, DNA demethylation and chromatin activation. In addition, we will use ChIP-qPCR assays to examine the proteins (MBD2, MeCP2: methylated DNA binding protein; H3K27me3: transcription repression; H3K4me2 & H3K27ac: transcription activation) that will be potentially associated with the promoter of gene of interest in VCaP, LNCaP, TRAMC1, and Pten-CaP2 and whether DIM and UA treatments will affect the association of these proteins leading to re-expression of these genes as we have done recently (128, 130). We will first focus on inflammatory and Nrf2-mediated anti-oxidative stress/anti-inflammatory genes in TRAMP C1 and Pten-CaP2 cells and inflammatory/cell cycle/death genes in VCap and LNCaP cells, which are relevant to prostate inflammation and prostate carcinogenesis and preventive effects of UA and DIM in Aims 1 & 2. In addition, as these epigenetic modifications may affect cell stemness, we will include aldehyde dehydrogenase isoform 1 (ALDH1) activity assay, which is a universal marker for somatic stem cells including the prostate.

***3e. To examine the regulation of microRNA expression by UA and DIM in TRAMP C1, Pten-CaP2, VCaP, and LNCaP cells:*** *Our Preliminary Studies show that DIM and UA down-regulated onco-miRNAs, such as miR18 and miR106, in LNCaP cells (**Fig. 12**). From the bioinformatics results of miRNA generated from Aims 1 & 2, candidate miRNAs will be confirmed in TRAMP C1, Pten-CaP2, VCaP, and LNCaP cells after treatment with UA and DIM for 24 hr, and subsequent molecular mechanisms, linking to inflammation, oxidative stress, apoptosis, and/or cell cycle arrest, will be investigated by knock-down and/or over-expression.*

***3f. Benchmarks for success, potential problems and alternative strategies for Aim 3:*** We do not anticipate any technical difficulties in execution of these experiments due to our prior experiences with the proposed experimental procedures (115, 128, 134, 139, 193, 194). It is possible that epigenetic alterations of the mouse and human inflammation and anti-oxidative stress genes could differ. For instance, silencing of GSTP1 expression via promoter methylation is a signature event in human PCa (199). However, GSTP1 appears not to be inactivated by methylation in the mouse (200). We will compare species-specific methylation patterns during inflammation response on PCa. In addition to the possible direct effect on DNA methylation, regulation of histone modification by polycomb group protein (PcG) is also an important mechanism for gene transcriptional repression. Previous report shows that EGCG treatment of SCC-13 cells reduces Bmi-1 and EZH2 level leading to reduce cell survival and a global reduction in H3K27me3 (201, 202). Furthermore, EZH2 was also reported to serve as a recruitment platform for DMNT (203). In collaboration with Dr. Pirrotta (204-206) [our collaborator and Internal Advisory Committee], we will consider investigating the effect of DIM and UA on PcG such as EZH2 and its relationship to transcription repression mark such as H3K27 as we proceed. The completion of our current study will discover potential molecular targets of histone modifying proteins (HATs, HDACs, HMTs, and KDMs) affected by UA and DIM which will enable the future research on the chemicals mechanisms as to how DIM and UA would affect these histone modifying proteins and DNMTs. It is postulated that similar to the electrophiles modification of sulfhydryl group seen in Keap1-Nrf2 signaling pathway (106, 107, 207), the role of electrophiles-GSH with subsequent conversion to electrophiles-Cys could potentially play a role in inhibiting HDACs (208) and similarly, green tea EGCG inhibits DNMT activity via competitive inhibition and molecular modeling studies show EGCG docks in the catalytic pocket of DNMT (209), will be enabled.

**Time Table for the proposed studies:**

The time table is constructed based on our previous experiences (134).

| | Year 1 | Year 2 | Year 3 | Year 4 | Year 5 |
|---|---|---|---|---|---|
| Aim 1 | ◎ | ◎ | ◎ | | |
| Aim 2 | | ◎ | ◎ | ◎ | |
| Aim 3 | | | ◎ | ◎ | ◎ |
| | Progress reports and manuscript preparation | | | | |

**Summary:** In this Project, we will test the hypothesis that inflammatory processes that would drive changes of inflammatory epigenome and CAM products would attenuate these inflammatory epigenomic alterations resulting in suppressing inflammation and its related diseases such as PCa in the prostate. We will utilize the selected experimental systems to address the common and the distinct epigenomic changes by closely work with the Analytical Core (attached in Appendix) on the NGS technology and the common analysis tools including bioinformatics, PK/PD and statistics. The intellectual connections will ensure and synergize the experimental design, streamline in common approaches, data analyses, result interpretations, and problem solving.

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