To the Graduate Council:

I am submitting a dissertation written by E-Chu Huang entitled “Mechanisms Associated with the Chemotherapeutic Effects of Zyflamend, a Multi-Herbal Extract, on Advanced Prostate Cancer”. I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutritional Sciences.

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Mechanisms Associated with the Chemotherapeutic Effects of Zyflamend, a Multi-Herbal Extract, on Advanced Prostate Cancer

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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E-Chu Huang

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I still remember the first time I started working on the project with Zyflamend® and was notified that I would be using a xenograft animal model of human prostate cancer. I had no clue what a xenograft model was or how to conduct this line of research. At the time, I was only familiar with cell culture techniques, but now I am very proficient at working with experimental rodent models after a lot of practice. “Practice, practice and practice” was always the golden

comment by Dr. Whelan and it is true.

In the last several years, I focused on identifying the mechanisms of Zyflamend® using different strategies and because of the nature of the product, it was not easy. The project was challenging because of the complexity of Zyflamend®. The concept of integrating many bioactive herbal extracts was novel and I had to do many experiments to explore a plethora of possible mechanisms. Developing a suitable hypothesis was an initial work-in-progress and at times frustrating. Fortunately, I had the opportunity to attend national and international conferences whose lectures helped inspire me. Eventually, I was able to systematically discover the multifaceted effects of Zyflamend® and build a wonderful story.

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Abstract

Advanced prostate cancer (PrC) is the second leading cause of death from cancer in US males. Advanced PrC cells are initially androgen-sensitive and thus androgen ablation therapy causes tumors to undergo regression and fall into a remission phase where residual cells remain dormant while androgen levels remain very low. Unfortunately, this phase usually lasts 3 to 5 years prior to tumor relapse, where the tumor cells re-grow in the absence of androgens. This form of the disease is aggressive and invariably fatal.

In this study, we investigated the effects of a combination of herbal extracts on various stages of PrC, androgen-dependent and castrate-resistant, using CWR22 and CWR22Rv1 cells, respectively. Zyflamend, a commercially available product consisting of 10 different herbal extracts, had been shown to reduce pre-malignant forms of PrC in clinical trials. We expanded these earlier experiments by using Zyflamend in a model of advanced PrC. Our initial results indicated that Zyflamend could repress androgen-sensitive and castrate-resistant (androgen-insensitive) prostate tumor growth. Using a cell model for castrate-resistant PrC, Zyflamend inhibited the growth of CWR22Rv1 cells by increasing the expression of the cell cycle inhibitors p21 and p27. These effects were mediated via hyperacetylation of histone 3 through the suppression of class I and II histone deacetylases (HDACs) and an induction of CBP/p300 histone acetyl transferase activity. The latter effect was mediated by the upregulation/activation of Erk-1/-2 and Elk-1. Zyflamend also inhibited androgen receptor expression, its downstream gene target, prostate specific antigen (PSA), and increased cell death by inducing apoptosis as indicated by caspase 3 activity and PARP cleavage. The reduction of androgen receptor was confirmed in CWR22Rv1 xenograft tissues. Our results suggest the extracts of this herbal

combination inhibits castrate-resistant prostate cancer cell growth epigenetically and by coordinately affecting androgen receptor signaling pathways involved in cell growth/death.

In an androgen-dependent PrC tumor xenograft model, Zyflamend reduced the growth of CWR22-derived tumors and enhanced the responsiveness of tumor cells to hormone ablation. Zyflamend potentiated the regression of PrC cells and their sensitivity to androgen deprivation. These results suggest that Zyflamend may be an effective adjuvant when used with hormone ablation therapy.

Keywords: prostate cancer, CWR22, 22Rv1, xenograft model, Zyflamend, HDACs, p21, androgen receptor

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Chapter I

Prostate Cancer

1. Introduction

Prostate cancer is the most commonly diagnosed solid malignancy and in the last few years the incidence of prostate cancer has risen dramatically and become the second leading cause of cancer-related deaths in men in the United States and most developed countries (1-3).

Progression of prostate cancer is a long process, and it often needs decades to develop into a clinically relevant disease. Prostatic intraepithelial neoplasia (PIN) is characterized by increased cellular irregularity and abnormal tissue morphology. Development of PIN takes ~twenty years, with another 10 years to progress to cancer (4). The incidence of prostate cancer increases dramatically from the fifth decade of life (5.3-14%) to the ninth decade (40-80%). The disease is diagnosed by monitoring the blood levels of prostate specific antigen (PSA) and with a tissue biopsy (5). At the early stages, the tumor is considered "low-grade" (Gleason grade 1-3). After three to fifteen years, early stages of the disease will progress to more advanced forms (Gleason grade 4-5) (6), eventually metastasizing to secondary sites, such as bone.

Metastatic prostate cancer can be defined by four phases. The first phase is androgen-dependent growth. In this stage, cancer cells are sensitive to the male hormone known as androgen and therapy often involves the removal of testosterone. Hormone ablation therapy by blocking the androgens/ androgen receptor activation leads to the regression of tumor. The second phase is characterized by regression following hormone deprivation. Following regression, the disease is considered to be in remission and this stabilization phase (Phase III) typically last between 3-5 years. During this time, blood PSA, a protein produced by prostate epithelial cells, is regularly measured to monitor the disease progression. The fourth phase is the androgen-independent phase. Tumor cells inevitably progress (relapse) in the absence of

androgens. The relapsed tumor is remarkably aggressive and death occurs within one to two years (Figure 1.1).

Epidemiologic studies suggest that multiple factors, including high fat diet, family history, age and high serum androgens, affect the incidence of prostatic cancer and its mortality (7, 2, 8). In this study, we are interested in the effects of dietary compounds on the advanced prostate cancer progression by blocking cell proliferation using androgen-dependent and androgen-independent prostate cancer cell lines. The dietary compounds used in this study are a mixture of herbal extracts, Zyflamend®; some of the herbal extracts have been shown to have anti-cancer properties *in vitro* and *in vivo* studies (9-15). Therefore, a combination of these herbal extracts might have a universal effect on inhibiting cancer cell growth through multiple pathways.

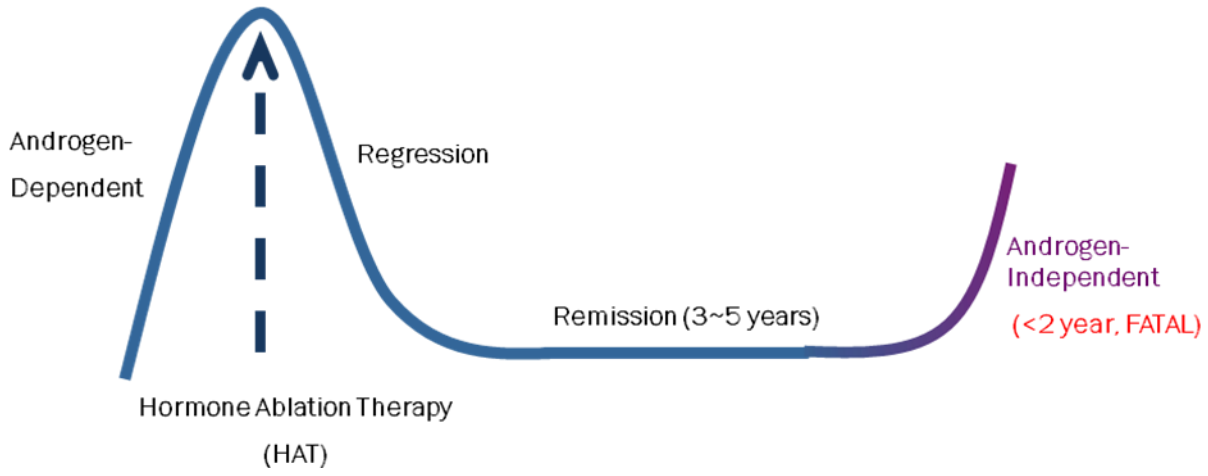

Figure 1.1 The Natural History of Advanced Prostate Cancer Cells Following HAT

1.1 Actions of androgen

In the eighteenth century, John Hunter was the first to reveal testicular function, and one hundred years later, Arnold Bechter reported the relationship between the behavior and physiological castration to a substance secreted by the testis into the plasma. In 1889, Charles Edouard Brown-Séquard isolated the extract derived from dog and guinea pig testis. Forty-five years later, Leopold Ruzicka and colleagues isolated the testicular hormone and named it testosterone. One year later, testosterone was artificially synthesized by Butenandt and Hanish (16). Nowadays, testosterone is one of many compounds in a group of male steroid hormones called androgens, including androstenedione, dehydroepiandrosterone and testosterone.

Androgens are important steroid hormones in males playing many physiological roles, including differentiation and maturation of the male sexual organs and contributing to male secondary sex characteristics (17). Androstenedione is produced in the adrenal glands and converted into testosterone in testis Leydig cells and peripheral tissue and it can be metabolized to estradiol by the action of aromatase. Dehydroepiandrosterone is produced in the adrenal cortexand is also synthesized de novo in brain (18). It is metabolized to androstenedione and androstenediol prior to their conversion to testosterone. Testosterone biosynthesis in testicular Leydig cells declines with age and these changes are related to increased expression of cyclooxygenase-2 and the decrease expression of steroidogenic acute regulatory protein (19) (20). The production of testosterone is regulated by feedback of luteinizing hormone (LH) and luteinizing hormone-releasing hormone (LHRH) of the gonad-hypothalamus-pituitary axis. Testosterone, the major circulating androgen in humans, diffuses into cells and is converted to 5α-dihydrotestosterone by 5α-reductase. In the cytoplasm, 5α-dihydrotestosterone binds to the

androgen receptor (AR) or a nuclear receptor (estrogen receptor) and further binds to hormone responsive elements such as androgen responsive elements and/or estrogen responsive elements to activate related gene transcription and stimulation of insulin growth factor- I (IGF-I) expression (21), and stimulating proliferation of prostate cancer epithelial cells (17).

In addition to androgen receptor, it is found that testosterone binding to a sexual hormone binding globulin (SHBG) or an androgen binding proteins (ABP) (22). A complex of testosterone and binding proteins bind to membrane testosterone binding sites and this association initiates a rapid action (seconds~minutes) involving endocytosis (23), and the actin cytoskeleton, ion-channels (TRPM8 (24)) and transporters affecting intracellular Ca^{2+} fluxes (25). This rapid association recruits heterotrimeric guanine nucleotide-binding proteins (G-proteins) and stimulates the downstreat signal transduction including PI3K, Ras and Src pathways (26-27).

In summary, the action of androgen signaling can be divided into two groups, direct steroid action, and indirect steroid action, described as the "Mannheim classification" (28). In the direct steroid action, membrane vesicles, fluidity and permeability as well as rapid responses would be changed without cell membrane receptors. In the indirect steroid action, the action of the steroids, requiring steroid receptors such as intracellular androgen receptors and SHBG receptors, mediate the activation of cAMP/PKA and induce AR and AR-related genes expression such as prostatic specific antigen (PSA) (29), and enhance phosphorylation of AR (30-31) and activation of Ras/Raf-1/Erk (32) and Ras/PI3K (33).

Whether androgen functions through an intracellular receptor (AR)-mediated pathway or through a rapid response-signal transduction pathway, they promote prostate cancer cell proliferation and tumorigenesis.

1.2 Hormone ablation therapy in androgen-dependent advanced prostate cancer

Androgen-dependent prostate cancer cells require the presence of androgens for growth. In 1941, Huggins and Hodges observed that malignant tumors arising from the prostate gland were partially responsive to the withdrawal of androgens and this led to the hormonal management of prostate cancer. This finding provided the solid basis for advanced prostate cancer treatment (34). Androgen deprivation therapy (ADT) is considered one of the first-line of treatments for men with high-grade, clinically localized prostate cancer who undergo radiotherapy (35) or metastatic prostate cancer (36). There are various methods to achieve testosterone ablation. Primary hormone ablation therapy includes maximal androgen deprivation (MAD) (also known as maximal androgen blockage, MAB). Maximal androgen deprivation refers to a combination of androgen suppression (AS) and anti-androgen therapy using luteinizing hormone-releasing hormone (LHRH) agonists to achieve medical castration and non-steroidal anti-androgens, such as flutamide, to inhibit tumor growth and increase 5-year survival rates (37).

Following primary therapy, the patients are monitored by measuring PSA levels as an indicator of tumor growth. If chemical relapse should occur (increasing PSA levels), patients could receive secondary hormone therapy. Secondary hormone ablation therapy 1) inhibits the production of adrenal androgens using corticosteroids, ketoconazole or liarozole and aminoglutethimide; 2) involves androgen receptor blockade using steroidal anti-androgens, such as cyproterone acetate, or non-steroidal anti-androgens, such as flutamide; 3) or involves estrogen therapy, using transdermal estradiol (38). Castrate-resistant prostate cancer (CRPC) is resistant to these therapies (39-41). Ultimately, men will develop a disease that is androgen-

independent /hormone refractory and eventually fatal.

1.3 Androgen-independent prostate cancer

Approximately 90% of advanced prostate cancer patients response to hormone ablation therapy (HAT); however, most still suffer tumor relapse within 5 years (42-43). Relapsed tumor cells can survive in the absence of androgens resulting in tumors that are unresponsive to treatment with an invariably fatal outcome. The mechanism of resistance to androgen withdrawal is being investigated. One of the mechanisms targets androgen signaling. During androgen deprivation, the loss of androgen appears to be a form of stress that may transform cells to derive from androgen-dependence to androgen-independent growth (44). There are several pathways leading to androgen-independent prostate cancer (in the absence of androgens). These pathways have been reviewed by Pienta KJ *et al*. (45) and Feldman BJ *et al* (46) and are summarized below.

1.3.1 The hypersensitive pathway

To survive in an environment of low levels of androgens, prostate cancer cells develop castration-resistance, as opposed to androgen independence, by increasing expression and sensitivity of androgen receptors, and by increasing 5α-reductase activity (47-48) to compensate for the low levels of androgens. Following hormone ablation therapy, 30% of recurrent prostate cancers have amplified androgen receptor gene expression with no amplification in primary tumor (49). In addition to an induction of androgen receptor, androgen receptor sensitivity is increased (lowering threshold) along with an increase in receptor stability (50), increasing the importance of local androgen production. After hormone ablation therapy, serum testosterone

levels decrease by 90%, but the level in prostate tissue is only reduced by 60% in compensation for the decline of testosterone in the circulation (47). The genes involved in steroid biosynthesis, such as HMG-CoA synthase, squalene synthetase, squalene mono-oxygenase, and lanosterol synthase have been found to be increased in recurrent human prostate carcinoma (51).

1.3.2 The promiscuous pathway

Most of the prostate cancer cells that are androgen-independent have been found to express androgen receptor. Some of prostate tumor cells develop amplified androgen signaling by increasing androgen receptor expression, androgen receptor sensitivity and local androgen production; other prostate tumor cells develop mutations on androgen receptors associated with alterations of co-regulators (52-53). Forty-four mutations of the androgen receptor have been identified, and twenty of those mutations had gain of function. Five of the twenty gain of function mutants had promiscuous activity that enhanced the activation of androgen receptor by non-androgenic steroid and anti-androgen molecules (52). The enhancement of androgen receptor promiscuous pathways can also be achieved through an induction of co-regulators such as cAMP response element-binding protein (CREB)-binding protein (CBP). The increasing androgen receptor activity and co-regulators promotes cancer cell survival against anti-androgen molecules (54).

1.3.3 The outlaw pathway

Androgen receptor signaling can be activated by outlaw receptors in a ligand-independent mechanism. IGF-1/IGF-1R has been shown to activate androgen receptor in the absence of androgen and induce prostate-specific antigen (PSA) expression (55). On the other hand, some

mutant androgen receptors are unable to bind DNA but can potentiate membrane tyrosine kinase signaling, such as activation of the Src-Erk1/2 pathway and induction of IGF-1R expression (56). Epidermal growth factor (EGF) has been found to activate the androgen receptor (55) and cytokines, including IL-6, has been shown to induce androgen synthesis (57).

1.4 The animal models of prostate cancer research

The development of prostate cancer takes many decades and the disease is very diverse in humans. This is a limitation in studying prostate cancer.. Therefore, identifying a suitable animal model would be a helpful tool to study this disease. The development of animal models of prostate cancer is important for the investigation of improved therapeutic strategies and to identify underlying molecular mechanisms in the event of effective intervention. Over the last 30 years, many rodent models have been developed, mimicking different physiological stages of prostate cancer progression. There are rodent models that study the disease as it forms spontaneously, or where prostate cancer is induced and xenograft models where human prostate cancer cells are grown subcutaneously in immune-compromised mice. Other models study pre-malignant forms of the disease (prostatic intraepithelial neoplasia, PIN), carcinomas, and metastasized tumors, in addition to models that are sensitive and insensitive to androgens (Table 1.1). These models show that 1) androgens are necessary for most prostatic carcinomas, 2) most advanced prostatic carcinomas eventually develop androgen-independence, and 3) advanced prostatic carcinomas metastasize to regional lymph nodes or distant organs such as bones and liver (58). However, it should be recognized that no single model can represent the diversity of the disease.

1.4.1 Rodent models

There are two major categories of rodent models when studying prostate cancer; one is the spontaneous rodent model and the other is the induced rodent model. Of the spontaneous rodent models, the Dunning rat model (59) is the most common and widely used model in nutritional studies (60). This model has identified some critical genes importatnt in the prostate, such as CD44. The Dunning R-3337 is derived from Dunning rat model and develops well differentiated adenocarcinomas. Sublines of Dunning R-3337 have a variety of characteristics of the disease being either highly metastatic or involving AR deficiency (61). ACI/Seg and Lobund-Wister rat models have also been used for nutritional studies for the progression of the disease. ACI/Seg rat model has a long lifespan with an age-related incidence of cancer, but the tumor does not metastasize. The Lobund-Wister rat model develops prostate adenocarcinomas that metastasize to the lung. This model has androgen-dependent properties in early stages of the disease but becomes androgen-independent thereaftere. In a N-methyl-N-nitrosourea (MNU)/testosterone propionate (TP) system, prostate adenocarcinomas can be induced by MNU, followed by a promotion with TP (62). Fisher F334 and Noble rats are rodent models whose prostate tumors are induced by carcinogens. The tumors formed in these two models are androgen dependent and non-invasive.

On the other hand, transgenic mouse models have the advantage of studying the effects of targeted genes on initiation, promotion, and progression of prostate cancer, providing a useful tool for therapeutics. For example, the TRAMP (transgenic adenocarcinoma mouse prostate) model has been used to study of the effects of post castration, oncogenes, and the role of IGF axis (63). TRAMP mice contain a rat probasin (PB), a nuclear protein expressed in the epithelial

cells of the rat prostate. PB is regulated by androgens, showing a similar PSA expression pattern as that of humans. The TRAMP model has a high incidence for the development of prostatic adenocarcinomas, with no metastasis. To study the progression from low grade PIN to high grade PIN, and development to invasive adenocarcinoma, C3(1)/SV40 transgenic mouse model containing a well characterized androgen-driven prostatic steroid binding protein (PSBP). This C3(1)/SV40 transgenic mouse model supports the idea that PIN is a precursor of carcinomas of the prostate (63).

Knockout mouse models provide an advantage when studying targeted gene alterations on prostate cancer progression. In patients with early stages of prostate cancer, deletions of *Pten* and *Nkx3.1* have been identified (64-65) and thus a heterozygous of *Pten* and *Nkx3.1* double mutation mouse model has been established. $Nkx3.1^{+/-}/Pten^{+/-}$ mice develop invasive adenocarcinomas accompanied by metastasis to lymph nodes after one year. After treatment of high-grade PIN with androgen ablation in these the mice, the prostate cancer cells exhibit androgen-independent properties (66-67) mimicking relapsed patients on hormone ablation therapy.

1.4.2 Xenograft model

The above models are based on rodent prostatic tumor cells, not human prostatic tumor cells. In order to study human prostate cancer in rodent models, athymic nude mouse xenograft models have been developed using cell lines derived from human prostate cancer. These human cells are derived from the tumors of prostate cancer patients and are transplanted into immune-deficient mice. A xenograph model using androgen-sensitive PC-82 cells (established in 1977) was the first prostate cancer xenograft model developed. Soon after, two androgen-independent

cell lines, PC-133 and PC-135, were established. (68). Since then, there are more than 25 xenograft models of human prostate cancer (61). In 1993, Pretlow's group from Case Western Reserve reported the initial use of the CWR22 prostate cancer cell line (69). This human cell line was derived from a primary tumor and is highly androgen dependent. When grown in a xenograph model, tumor regression occurs following androgen deprivation. Relapses to an androgen-independent line, CWR22R occurs in 30-50% of the animals within 3-10 months of castration (69). Microarray analysis has been identified changes (cutoff >2.5 fold) between CWR22-derived androgen-dependent tumors, and the CWR22R-derived androgen-independent tumors. Thirteen genes that increased were associated with thyroid hormone receptor signaling and cell surface receptors; 44 genes were decreased (70). In addition, mutations of hAR signaling of the CWR22 cell line has been reported with a mutation in the ligand-binding domain of the hAR gene which can be transcriptionally activated by testosterone, dihydroxytestosterone (DHT), dehydroepiandrosterone (DHEA), estradiol, progesterone, and the anti-androgen hydroxylflutamide (71). This AR mutation is found in relapsed prostate cancer patients, and in the CWR cell line series of androgen-independent xenograft models (CWR22, CWR22R, and CWR91). As such, these models have been used for the evaluation of drug therapy (72).

Other xenograft models can be generated using various cell lines, such as androgen-dependent LNCaP cell line and androgen-independent PC3 and DU145 cell lines. These models are widely used to study the androgen dependent and androgen-independent stages of prostate cancer. The properties of these cells are discussed in the next section.

Table 1.1 Rodent models of prostate cancer (66-67, 59, 73-74, 60, 58, 62, 61, 63, 68)

Model	PIN/CaP pogression	Metastasis	Androgen status	Features of cancer
Spontaneous rodent model				
ACI/Seg rat	>33 mo	No	ND	30-40% develop to invasive adenocarcinoma
Lobund-Wister (Pollard) rat	>26 mo	lung	AD in early stage; then AID	High grade PIN
Dunning rat	2 mo	lung	AID	rapid growth and rapid metastasis
Induced rodent model				
Lobund-Wister (Pollard) rat	10.5 mo	lung, liver	AD in early stage; then AID	three types of cancer develop: moderately to poor differentiated prostatic adenocarcinoma, hepatocellular carcinoma, and lung carcinoma
Fisher F334 rat	20 wk	No	AD when DMAB followed by TP; AID when DMAB+TP	moderately differentiated noninvasive cancer
Noble	44 wk	rare	AD	100% of rats develop PIN and then CaP by testosterone treatment for 16 wk and for 44 wk, respectively
Wistar rat	2 mo	lymph node, lung, rare to bone	AD	10% incidence of metastasis in one yr

ND: no determined; NE: neuroendocrine; AD: AR dependent; AID: AR independent; WT: wild type; DMAB: dimethylbenz(a)anthracene; TP: testosterone propionate

Table 1.1 Continued.

Model	PIN/CaP pogression	Metastasis	Androgen status	Features of cancer
Transgenic mouse model				
Probasin- SV40 T antigen (TRAMP)	7-12 wk	lymph node, lung, kidney, bone, adrenals (28 wk)	variable	progressive disease restricted to the prostate epithelium; metastatic adenocarcinomas are partially androgen-independent
Large probasin-SV40 T antigen	10-22 wk	No	AD	invasive adenocarcinoma
Fetal Gγ globin	16-20 wk	lymph node, lung, kidney, bone, thymus, adrenals (24 wk)	AID	progressive prostate disease with epithelial and NE feature
C3(1)-SV40 T antigen	8-28 wk	rare	ND	invasive adenocarcinoma
C3(1)-polyomavirus middle T	11 wk	No	ND	invasive adenocarcinomas
Cryptdin-SV40 T antigen	8-12 wk	lymph node, liver, lung, bone (24 wk)	AID	NE-derived progressive prostate disease
Pten, *Nkx3.1* mutant mice	6 mo	lymph node	AID lesions with WT AR	HGPIN/early carcinoma lesions
Xenograft model-CaP cell lines	**Origin**	**AD/AI**	**AR**	**PSA**
CWR22	prostate	AD	mutant	positive
LAPC-3	prostate	AID	WT	positive
LAPC-9	bone	AD	WT	positive
LuCaP 23.1	lymph node	AD	N/A	positive
LuCaP 23.12	liver	AD	N/A	positive
MDA Pca-31	liver	N/A	positive	positive
PC-82	prostate	AD	WT	positive
PC-329	prostate	AD	WT	positive
PC-374	skin	AID	WT	positive

1.5 The cell model of prostate cancer research

To study the underlining mechanisms of prostate cancer progression, cell models have been developed that mimic various stages of prostate epithelial cells, normal and neoplastic, including those cells that are androgen-dependent and androgen-independent (Table 1.2). Different cell lines are thought to represent different characteristics of the disease. For example, a mutation of the androgen receptor has been found in LNCaP cells and this mutation results in the substitution of alanine for threonine at position 877 (T877A) (75). The same mutation was observed in metastatic tumor tissues from six out of twenty-four patients (76) and was also observed in bone marrow metastatic tissues from 5 out of sixteen patients receiving combined androgen blockage and flutamide (77), indicating this mutant form of the androgen receptor is common in patients. Therefore, LNCaP cells can potentialy become androgen independent. The use of LNCaP cells to mimic the transition of androgen dependence to androgen independence has been widely used to study the signaling mechanisms and genotypes during prostate cancer progression after androgen deprivation.

MDA PCa 2b is an androgen dependent cell line that was derived from a tumor that metastasized to bone. MDA PCa 2b cells have an androgen receptor mutation (T887A and L701H) and this mutation reduces the binding affinity of androgen. Although MDA PCa 2b cells are less sensitive to androgens, they can still respond to androgens and produce PSA (78).

Similarly, androgen receptor mutation was also found in CWR22 cells at position 874 (substitution of tyrosine for histidine) in the ligand binding domain at c-terminus. This mutation influences the binding of co-regulators and its transcriptional activity (79).

PC3 and DU145 cells are the most common cell lines used to study the mechanisms of androgen-independent cell/tumor growth. However, these two cell lines do not express an androgen receptor and do not produce PSA, two key components associated with advanced and metastatic prostate cancer. It is believed that expression/activation of androgen receptor plays an important role in the resistance to hormone ablation therapy and contributes to the transformation of androgen-dependent to androgen-independent cell growth. Therefore, the use of these two cell lines are unable to address the role of the androgen receptor in the disease.

CWR22Rv1 cells are derived from relapsed CWR22 cells and are castrate-resistant cells. CW22Rv1 cells can survive in the absence of androgen but can still respond to androgen stimulation. Androgen receptor is expressed by CWR22Rv1 cells and PSA is produced in the presence of androgen. CWR22Rv1 cells express two isoforms of androgen receptor; one is a 114-kDa protein and other one is a 75~80-kDa protein. The two isoforms have been identified. The 114-kDa protein has a duplication of exon 3 encoding zinc finger domain of the androgen receptor DNA-binding site, while the truncated protein lacks the ligand-binding domain (LBD) in COOH-terminus, referred to as ARΔLBD (80), these two isoforms do not exist in parental CWR22 cells. The in-frame androgen receptor regulates endogenous androgen-dependent gene expression and the truncated androgen receptor promotes cell proliferation in a ligand-independent manner. The two isoforms of androgen receptor is constitutively expressed and are active following hormone deprivation. Because of these characteristics of androgen receptors expressed in CWR22Rv1 cells, CWR22Rv1 cells have advantages when studying the progression of prostate cancer during the refractory state and can serve as a model to understand the mechanism of transition from androgen-dependent to androgen-independent cell growth. As

such, CWR22Rv1 cells were the cell model of choice for the *in vitro* and *in vivo* studies in this project.

Table 1.2 Cell models of human prostate cells

human cell line	ATCC#	disease	tumorigenic	source	AR	AD/ AID	PSA
immortalized prostate cells							
RWPE-1	CRL-11609	normal	No	epithelial	+	AD	+
RWPE-2	CRL-11610	normal	Yes	epithelial	+	AD	+
PWR-1E	CRL-11611	normal	No	epithelial	+	AD	+
PZ-HPV-7	CRL-2221	normal	No	epithelial	+	AID	-
WPE1-NA22	CRL-2849	normal	Yes	epithelial	+	AD	+
WPE1-NB14	CRL-2850	normal	Yes	epithelial	+	AD	+
WPE1-NB11	CRL-2851	normal	Yes	epithelial	+	AD	+
WPE1-NB26	CRL-2852	normal	Yes	epithelial	+	AD	+
RWPE2-W99	CRL-2853	normal	Yes	epithelial	+	AD	+
WPMY-1	CRL-2854	normal	No	stroma	+	AD	+
WPE-stem	CRL-2887	normal	No	peripheral zone	+	AID	+
WPE-int	CRL-2888	normal	No	peripheral zone	+	AD	+
WPE1-NB26-64	CRL-2889	normal	Yes	peripheral zone	+	AD	+
WPE1-NB26-65	CRL-2890	normal	Yes	peripheral zone	+	AD	+
prostate cancer cells							
LNCaP	CRL-1740	carcinoma	Yes	left supraclavicular lymph node	+	AD	+
CA-HPV-10	CRL-2220	adenocarcinoma	No	epithelial	+	AD	+
MDA PCa 2b	CRL-2422	adenocarcinoma	Yes	bone	+	AD	+
VCaP	CRL-2876	cancer	Yes	vertebral metastasis	+	AD	+
NCI-H660	CRL-5813	small cell carcinoma		lymph node	ANPR	AID	N/A
PC-3	CRL-1435	adenocarcinoma	Yes	bone	-	AID	-
DU-145	HTB-81	carcinoma	Yes	brain	-	AID	-
22Rv1	CRL-2505	carcinoma	Yes	epithelial	+	AID	+

AD: AR dependent; AID: AR independent; ANPR: atrial natriuretic peptide receptor

1.6 Cellular mechanisms of prostate cancer

The progression of prostate cancer is a long process and the mechanism is complicated. The mechanisms involved in tumorigenesis include promotion of cell growth, metastasis and angiogenesis, as well as inhibition of cell death. The regulation of proliferative mechanisms is influenced by the surrounding environment, such as factors in circulation, i.e., cytokines and growth factors, such as IGF-1 and androgens, and the influence of nearby tissues, i.e. fibroblast, and other stimuli, i.e. hypoxia. These mechanisms are discussed as below.

1.6.1 Cell growth

Cancer progression is associated with the deregulation of cell growth and cell death. In cancer cells, the signal transduction of cell proliferation is up-regulated. For example, cell cycle promoting regulators such as cyclin dependent kinases (CDKs) are induced and cell cycle suppressors such as the retinoblastoma (Rb) family and p53 are inhibited, thereby continuing cell division. In addition, cell death signal pathways are inhibited and thus cancer cells escape apoptosis, and the growth of cancer cells is promoted. To support tumor cell growth, formation of new blood vessels provide sufficient nutrients and oxygen for survival and channel cells from local areas to more distance sites, i.e. bone, in most prostate cancer patients (81).

1.6.1.1 Cell cycle arrest

Cell growth and division is tightly regulated by cell cycle progression. Cell cycle stages are defined as Gap 0 (G0), Gap 1 (G1), Synthesis (S), Gap 2 (G2) and Mitosis (M) phases. In the G0 phase, a resting and quiescent phase, progression through cell cycle and

cell division is halted and for transition to the G1 phase. In the G1 phase, the cell is enlarged and the production of mRNA and protein is increased in preparation for DNA synthesis. There is an important cell cycle mechanism regulated during this period which is G1 checkpoint. G1 checkpoint, a point of transition of the G1 and S phases, decides whether the cell continues to the S phase or remains resting in the G0 phase. In tumor cell progression, the G1 checkpoint is a critical regulatory stage. G1 checkpoint is negatively regulated by cyclin dependent kinase inhibitors (CDKIs) including Cip/Waf family (p21, p27 and p57) and INK4 family (p15, p16, p18, p19) and by tumor suppressor genes such as retinoblastoma (Rb) and p53. Members of the Cip/Waf family bind cyclin/cyclin dependent kinase complexes and disrupt the catalytic activity of CDKs (CDK4/6 and CDK2) on cyclins (cyclin D and cyclin E). Disruption of CDKs inhibits DNA polymerase, thymidine kinase and hydrofolate reductase, all required for DNA synthesis in the S phase. The Rb family is another major regulator of the G1 phase through the Rb/E2F pathway. E2F is a transcription factor that binds the promoter region of many genes, such as DNA polymerase subunits, cyclin A and cyclin E, allowing the initiation of DNA synthesis and progression to the S phase. E2F is inhibited by Rb to form a Rb/E2F complex in the cytosol; however, E2F is released from the complex when Rb becomes hyper-phosphorylated by the cdk–cyclin complex (82). In prostate cancer patients, most of the CDKIs, p21, p27 and p16 and Rb, are repressed by genetic and epigenetic regulation (83).

The second checkpoint of the cell cycle is at S phase where the cells replicate DNA. The S phase checkpoint is regulated through ataxia telangiectasia mutated (ATM)/ATM and Rad3-related (ATR)-checkpoint1 (chk1)-cell-division-cycle 5A (cdc25A) (ATM/ATR-chk1-cdc25A) and ATM-Nijmegen breakage syndrome1 (Nbs1) - Structural maintenance of

chromosomes protein 1 (SMC1) (ATM-Nbs1-SMC1) pathways. DNA damage stimulates phosphorylation of Ser/Thr kinases, checkpoint kinases 1 and 2 (chk1 and chk2), by ATM or ATR. Activated chk1/2 phosphorylates the serine sites of cdc25A phosphatase, the inactive form, thereby reducing the activity of cyclin E and Cdk2. The inactive cyclin E/Cdk2 is incapable of phosphorylating Cdc45, disrupting DNA replication (84). ATM-Nbs1-SMC1 also regulates the S phase checkpoint. This pathway is induced by ionizing radiation. ATM phosphorylates SMC1 at S957 and S966 sites, which recruits a phosphorylated Nbs1 as an adaptor (85). The ATM-Nbs1-SMC1 pathway is distinct from the ATM/ATR-chk1-cdc25A branch, but it is still required for the activation of the S phase checkpoint.

The third checkpoint is G2/M checkpoint initiating the DNA repair before entering into the mitosis. Activation of the G2/M checkpoint is induced by ATM/ATR-chk1-cdc25A. cdc25A phosphatase is inactivated by chk1/2 and binding to 14-3-3 proteins. The 14-3-3/cdc25A complex translocates into the cytoplasm followed by degradation. Therefore, the downstream complex, cdk1/cyclin B1, cannot be activated and the structure of chromosome remains tight, arresting the cell cycle (86).

Checkpoints deregulate cell cycle progression and allow unlimited cell proliferation. Most cancer treatments focus on enhancing the function of the checkpoints through radiation therapy or deprivation of hormones or chemotherapies, thereby inducing DNA damage and activating ATM/ATR pathways, as well as inducing expression of cell cycle inhibitors resulting in cell cycle arrest.

1.6.1.2 Cell apoptosis

Cell apoptosis was termed “programmed cell death” (PCD), which is a form of cell suicide. The mechanisms of programmed cell death have been identified and include autophagy, paraptosis, mitotic catastrophe and apoptosis (87). Apoptosis is more specifically defined as a progress of cell death mediated by caspases through intrinsic and extrinsic pathways. Apoptosis occurs following the removal of growth factors or growth stimulating hormones, i.e. fibroblast growth factor or androgens, and the exposure of cytotoxic chemicals or pro-apoptotic ligands, such as TRAIL (TNF-related apoptosis-inducing ligand), activating the caspase cascade.

Caspase cascade includes intrinsic or extrinsic pathways (88). The intrinsic pathway, also called the mitochondrial pathway, and is initiated by intracellular stress, DNA damage. Double-strand DNA damage stimulates ATM (ataxia-telangiectasia-mutanted) activation and induces the expression of the tumor suppressor gene p53. p53 is an important negative regulator of the cell cycle and promotes the expression of pro-apoptotic proteins (e.g. puma, Bax, apaf-1, FasL and DR5) (89). Puma (p53 upregulated modulator of apoptosis, a member of pro-apoptotic Bcl-2 family) inhibits the association of anti-apoptotic protein Bcl-2, Bcl-x and pro-apoptotic protein Bax and Bak. Therefore, free Bax and Bak translocate to the outer membrane of the mitochondria, enhancing the release of cytochrome c and Smac/DIABLO (Second Mitochondria-derived Activator of Caspases/Direct IAP Binding Protein with Low PI) into the cytoplasm (90). Cytochrome c, Apaf-1 and pro-caspase 9 form an apoptosome, a complex of proteins formed in the process of apoptoisis. In the apoptosome, pro-caspase 9 becomes active by a conformation change and activates the downstream effectors caspase 3,

6 and 7 and IAPs (inhibitors of apoptosis). Smac/DIABLO inhibits IAPs through physical interaction, and therefore negatively affects caspase activity (91). Active effectors (caspase 3, 6 and 9) initiate the death substrates, PARP and Gas2 (92), resulting in apoptosis.

The extrinsic pathway is induced by apoptosis-induced ligands, such as FasL/CD95L and TRAIL, binding to the pro-apoptotic membrane receptors and decoy receptors. Once activated, the death domain of the receptor recruits the adapter molecules such, as FADD (Fas-associated death domain) and the initiators, pro-caspases 8 and 10, forming a DISC (death-inducing signaling complex). By proteolytic processing, initiator caspase 8 and 10 is active and activates caspase 9 following the activation of caspase 3, 6 and 7. Initiator caspase 8 and 10 cleave Bid to form tBid (truncated Bid). tBid associated with Bax and Bak forms a channel on the mitochondrial outer membrane (MOM) and increases MOM permeabilization, releasing pro-apoptotic proteins, such as cytochrome c. Intrinsic apoptosis is initiated (90, 93).

1.6.2 Metastasis

Metastasis is a process whereby cancer cells relocate to the secondary sites, such as lymph nodes, lung or bone. In this process, it is involved in angiogenesis, migration and invasion. While the tumor mass is increasing, angiogenic molecules are generated by the tumor cells and stromal cells to stimulate new vessel formation. The tumor cells change their morphology and secrete proteinases to pass through the surrounding barrier and enter into the circulation. Eventually, tumor cells spread to the other organs.

1.6.2.1 Angiogenesis

Angiogenesis is a process of new blood vessel formation. In tumors, new vessel formation provides a mechanism to supply sufficient nutrients, oxygen and pro-tumorigenetic factors to the cancer cells and to remove waste form the cell cluster. In addition, new vessel formation is critical for tumor metastasis. During tumorigenesis, angiogenesis occurs in different stages of tumor development, including premalignant lesions and malignancy, and allows tumor progression, thereby increasing tumor mass. This process is initiated by angiogenic molecules generated by the cancer cells and also by the stroma under environmental stimuli, such as hypoxia. Angiogenic molecules include vascular endothelial factor (VEGF), platelet-derived growth factor (PDFG), placental growth factor (PlGF), angiopoietin-1 (ANG1) and basic fibroblast growth factor (bFGF). VEGF is one of the most ubiquitous molecules for angiogenesis and released not only from the cancer cells, but also by fibroblasts and inflammatory cells such as macrophages. VEGF has an important role in new vessel formation by promoting the growth of vascular endothelial cells (ECs) and is also a survival factor for ECs by preventing cell death (94). The expression of VEGF is upregulated by various growth factors, including insulin-like growth factor-1 (IGF-1), PDGF and bFGF, suggesting that the release of VEGF is coordinately regulated by local hypoxia and growth factors in the microenvironment (95).

In prostate cancer, VEGF and endoglin (a protein that is involved in vascular remodeling) expression were detected in 50 radical prostatectomy specimens, categorized by Gleason score. VEGF correlated significantly with angiolymphatic invasion and Gleason score ($P < 0.05$) and endoglin correlated positively with Gleason score, lymph node metastases, tumor

stage, and preoperative prostate-specific antigen level ($P < 0.05$). Expressions of VEGF and endoglin were correlated with shorter survival times (96). In addition, serum levels of VEGF were significantly higher in the metastatic prostate cancer patients compared to patients with localized tumors (97). These results indicated that the pro-angiogenic molecule, VEGF, was associated with prostate cancer cell metastasis and progression.

One of the activators of VEGF is hypoxia-inducible factor 1α (HIF-1α). HIF-1α is a transcription factor and is induced by hypoxia while the tumor mass is increasing. Under a low oxygen environment, the repressor of HIF-1α, von Hippel-Lindau (VHL), is released from the HIF-1α-VLH complex (98), resulting in activation of HIF-1α, allowing it to bind to genes such as VEGF. Overexpression of HIF-1α was observed in prostate malignancy and non-malignant prostate tissues compared with the normal prostate (99). This is consistent with elevated expression of VEGF in prostate cancer and increases in the degree of neovascularization with tumor stage.

1.6.2.2 Migration and Invasion

The translocation of tumor cells as a result of the formation of new vessels, angiogenesis, requires the ability to pass through a multilayer barrier composed of pericytes, base membrane, fibroblast and endothelial cells, and is referred to as migration and invasion. Tumor cells elongate polarize and stiffen while interacting with the surrounding environment, such as extracellular matrix (ECM) substrates, i.e., collagen, elastin and fibronectin. Tumor cells, macrophages and fibroblast can secrete ECM-degrading proteases, including urokinase-type plasminogen activator (uPA) and the matrix metalloproteinases (MMPs) such as MMP2, MMP7,

MMP9, and MMP12 (100), resulting in ECM degradation and providing space for cell invasion into the underlying tissue and migration into the circulation.

Tumor cells in the circulation would be attracted by certain growth factors and move to the secondary site.This interaction between tumor cells and target tissues are hypothesized as "seed and soil". Briefly, "seed and soil" hypothesis was first published in 1889 by English surgeon Stephen Paget where tumor cells are the seed and the target tissues are the soil. He analyzed more than 900 autopsy records and found that tumor cells metastasize to specific organs (101). His observations suggested that sites of metastasis are determined by both the characteristics of those tumor cells and the microenvironment of the host tissue (102). In prostate cancer, 90% of patients with hematogeneous metastases develop bone metastasis (81), suggesting that bone marrow-derived cells (VEGF receptor-1 and -2 positive progenitor cells) and bone-specific matrix in the tumor microenvironment promotes and attracts metastasis to bone (103). This interaction between local prostate cancer cells and bone marrow is consistent with the "seed and soil" hypothesis (104). In addition, several growth factors such as IGF-1 produced by bone marrow cells promote prostate cancer growth and survival through a stimulation of the IGF-1/IGF-1R survival signal pathway.

1.6.3 IGF AXIS

Insulin-like growth factors (IGFs) are polypeptide growth factors secreted from many tissues with functional homolgy to insulin. There are two types of IGFs, IGF-I and IGF-II, sharing 62% homolgy in their amino acid sequence (105) and tightly regulated in the circulation by insulin-like growth factor binding proteins (IGFBPs). Both IGF-I and IGF-II bind to IGF-I receptor (IGF-IR), a transmembrane tyrosine kinase receptor. IGF-IR is a tetramer of two

identical α-subunits and two β-subunits. IGF-IR can resemble the insulin receptor forming a hemireceptor and binds to IGF-I, but not insulin (106). IGF-I/IGF-IR binding presents a similar signal transduction pathway as the insulin signaling cascade. Over 99% of the circulating IGFs are bound to IGFBPs, with a majority forming a complex with IGFBP-3 and the acid-labile subunit (ALS). The binding of IGF-I to this complex prolongs its half-life in the serum. IGFs have a greater binding affinity with IGFBPs than IGF-I receptors, therefore regulating bioactivity of IGF and signaling at the cellular level via affinity competition (107). However, the function of IGFBP is very controversial. In the CWR22 human prostate cancer xenograft model, it has been shown that IGFBP-5 was regulated by androgen receptor (AR), whereby the interaction of AR with IGFBP-5/IGF was associated with tumor growth (108). On the other hand, PSA, an androgen-related gene, induced IGFBP-5 degradation, enhancing IGF/IGF-IR interaction and IGF-1R phosphorylation., Activation of the IGF-1R signaling pathway promotes malignancy progression (109). IGF-IIR, on the other hand, has no tyrosine kinase activity and selectively binds to IGF-II. This process results in the degradation of IGF-II and controls circulating levels.

1.6.3.1 IGF-IR signal in cell proliferation

IGF-I/IGF-IR signaling also modulates cell cycle regulation. Activation of PI3K/AKT by IGF-I/IGF-IR signaling phosphorylates p70 S6K and elF-4E. Phospho-elF-4E associates with ribosome and thus accumulated ribosome becomes a sufficient translational machinery to ensure the rapid processing of transcripts through the cycle (110). Activation of IGF-I/IGF-IR increases the release of E2F from retinoblastoma (Rb). It also increases synthesis of cyclin D1, cyclin E and CDK4 through MAPK (Erk) activation (111) (112), and reduces the expression of the cell cycle inhibitors p21 and p27 (113). Collectively, these events promote the transition of the cell

cycle from G1 to S phase. The production of cyclins A and cdc2 are induced by IGF-I/IGF-IR signaling and thereby enhance cell cycle progression from the G2-M phase (114). Taken together, IGF-I/IGF-IR activation promotes cell cycle at several phases, mainly through AI3K/AKT and MAPK pathways, and thereby inducing cell proliferation.

1.6.3.2 IGF-IR signal in apoptosis

Downstream of IGF-I/IGF-IR signaling cascades are AKT and MAPK intracellular signaling. The IGF-1R activation leads to autophosphorylation on tyrosines 1131, 1135 and 1136 in the kinase domain, followed by phosphorylation of juxtamembrane tyrosines and carboxy-terminal serines and subsequent phosphorylation of insulin receptor substrate (IRS) proteins (115). Signal transduction proceeds through IRS with the activation of PI3K/AKT and MAP kinases and involves phosphorylation of downstream substrates including the members of Bcl-2 family, Bad and forkhead transcription factors (i.e., FKHRL1), and caspases (107, 116). Phosphorylated Bad binding to 14-3-3 proteins relocates into cytosol and neutralizes the pro-apoptotic function of Bad (117-118). In addition, AKT activation inhibits the caspase cascade by phosphorylation of caspase 9 and caspase 3 (119). IFG-IR is frequently overexpressed in carcinomas of breast, lung, colon, and prostate (120) and overexpression of IGF-IR reduces p53-mediated apoptosis through a p53-Mdm2 feedback loop (121). In addition, IGF-IR signaling inhibits apoptosis signal-regulating kinase 1 (ASK1) which is a MAP kinase kinase kinase (MAPKKK) that is required for c-Jun N-terminal kinase (JNK) and p38 activation in response to Fas and tumor necrosis factor (TNF) receptor stimulation in the extrinsic apoptotic pathway. IGF-IR forms a complex with ASK1 and suppresses ASK-1-mediated stimulation of JNK/p38 and the induction of programmed cell death in a PI3K independent manner (122). The presence

of multiple anti-apoptotic pathways may explain the efficacy of the IGF-IR in protecting cells from apoptosis.

1.6.3.3 IGF-IR signaling in cell angiogenesis and metastasis

Angiogenesis (neovascularization) is a process by which new blood vessels are formed from current blood vessels. This process is critically important for the growth and development of tumors. As tumors enlarge, centrally located cells require sufficient oxygen and nutrients for viability and the removal the waste during metabolism. In addition, it creates a new path for cells to migrate to more distance organs. IGF-IR has been shown to increase VEGF expression through an induction of hypoxia-inducible factor 1α (HIF-1α) via PI3K and MAPK pathways (123), and therefore promotes endothelial cell proliferation. IGF-IR also cooperates with integrins, αvβ3, and activates PI3K and MAPK signals in promoting angiogenesis and cell metastasis (124-126). Angiogenesis is an obligate requirement for tumor cell invasion, migration,, and metastasis.

Along with promoting angiogenesis, IGF-IR signaling contributes to the metastatic process by affecting cell behavior, adherence, motility, and invasive ability. Cell-cell interaction is tightly regulated by adhesion molecules such as E-cadherin, β-catenin and integrins. Activation of PI3K/AKT via IGF-IR signaling has been shown to reduce epithelial markers, E-cadherin and β-catenin, and therefore promote IGF-IR-induced cell motility (127). The reduction of cell adhesion breaks down intracellular adherent junctions and changes cell behavior from an epithelial cell type to a mesenchymal cell type. This process is called “epithelial-mesenchymal transtion” (EMT). This is a classical transition when tumor cells are transformed to undergo migration and invasion. In addition, IGF-I induces cell migration by promoting MMP production

and thus promoting a degradation of the sorrounding extracellular matrix (ECM) (128), and establishing a proper microenvironment for cell migration and invasion. MMPs have to be activated by a proteolytic process and IGF-IR/PI3K/AKT promotes MMP protein activation by increasing a proteolytic activator. MMP-14 is one of the proteolytic activators of MMP-2 (129). MMP-2, MMP-7 and MMP-9 have been shown to free IGF via proteolytic cleavage of IGFBP3 (130-131), increasing IGF bioactivity and constitutively activating IGF-IR signaling.

1.6.3.4 Epidemiological studies of IGF/IGF-IR axis in prostate cancer

There is a positive correlation between the IGF signaling axis and prostate cancer. The risk for prostate cancer was more than 4 fold higher in men with the highest circulating levels of IGF-I (highest quartile) compared to the men in the lowest quartile (132). In addition, plasma levels of IGF-I and IGFBP3 were the predictors of advanced prostate cancer (133). In a nested case–control study within the Northern Sweden Health and Disease Cohort Study, subjects with prostate cancer had significantly higher mean levels of IGF-I and IGFBP-3 compared to controls (134). Furthermore, a higher ratio of IGF-I:IGFBP3 was associated with increased risk for benign prostatic hyperplasia (BPH) (a potentially preneoplastic condition), while high serum IGFBP3 levels were associated with decreased BPH risk (135). In addition, IGF-I levels and IGF-IR expression increased significantly as the prostatic tissue transitioned from BPH to PIN to malignancy, demonstrating a high correlation between the IGF axis and prostate tissue morphology and tumor grade (136). These observations were supported by data showing stroma adjacent to Gleason grade ≥7 tumors had higher expression of IGF-IR than the lower grade tumors and benign epithelium (137). Chen et al. analyzed 96 studies and over 110,000 subjects for the relationship between circulating levels and polymorphisms of IGF1 and IGFBP3 and

cancer risk (138). They found increased levels of IGF-I significantly increased cancer risk by 15%, whereas increased IGFBP3 levels significantly decreased the risk of advanced prostate cancer by 56%. Taken together, circulation of IGF and IGFBP as well as the expression of IGF-IR may serve as biomarkers for prostate cancer development.

Controversially, some evidence showed that the IGF axis disrupts hormone ablation therapy on prostate cancer patients. Before hormone ablation therapy, the IGF-I level was correlated with bone metabolic disorder, but there was no relationship after hormone ablation therapy (p=0.565). This result indicated that IGF axis may be inactivated during hormone ablation therapy (139). The role of IGF-1/IGF-1R axis should be clarified.

1.6.4 HDACs mechanisms

Histone deacetylases (HDACs) are a family of proteins that are involved in cancer development and have been a target for cancer therapy. There are several members in the HDAC family and can be classified as class I, class II, class III and class IV. The class III subgroup is also called known as sirtuins and depend on nicotinamide adenine dinucleotide (NAD1) for their catalytic activity, exhibiting catalytic activities similar to ADP-ribosyltransferases or ADP-cyclases. Some of the Class III members lack deacetylase activity.

Histone deacetylases of class I, class II and class IV are zinc-dependent amidohydrolases and represent the classical activity of deacetylases. The function of HDACs is activated through a zinc-based catalytic mechanism. At the transcription level, HDACs cleave acetyl groups from lysine residues on histones, particularly histone H3 and H4. The removal of acetyl group condensed the chromatin structure and decreases gene expression, epigenetically. Acetylation of

histones are involved in chromatin assembly (140), DNA repair and recombination (141). Therefore, accumulation of epigenetic changes results in the alteration of gene expression.

Histones are not the only substrates of HDACs, and growing evidence suggests that HDACs function on the non-histone proteins such as p53 (142) and IRS-I (143). In addition, non-protein molecules such as polyamines or metabolic intermediates might also be substrates of HDACs (144).

Both acetyaltion and deacetylation have different effects on proteins. First, acetylation or deacetylation on select lysine residues can affect protein stability by site competition for ubiquitylation (145), protein-protein interaction (changes in configuration), and protein localization. For example, deacetylated Ku70, a nuclear DNA-damage-response protein, complexes with Bax, a pro-apoptotic protein, protecting cells from apoptosis. Bax dissociates from acetylated Ku70 and translocates into mitochondria to activate intrinsic apoptosis (146).

Activity of classical HDACs is inhibited by zinc chelators, mainly thiols or hydroxamic acid derivatives like TSA (trichostatin A) and SAHA (suberoylanilide hydroxamic acid, INN: Vorinostat) (147). An increasing number of studies show that inhibition of HDACs suppress tumorigenesis by increasing cell death and inhibit cell proliferation by altering the acetylation status of chromatin and other non-histone proteins (Figure 1.2). Inhibitors of class I, class II and class IV HDACs have been identified. Most of them are derived from the natural compounds and some of them are artificially synthesized. These HDAC inhibitors have been used in the phase I, phase II and phase III clinical trials on hematological malignancies and various solid tumors (148-149). The combination of HDAC inhibitors with other anticancer agents associated with traditional therapy may be a favorable clinical treatment.

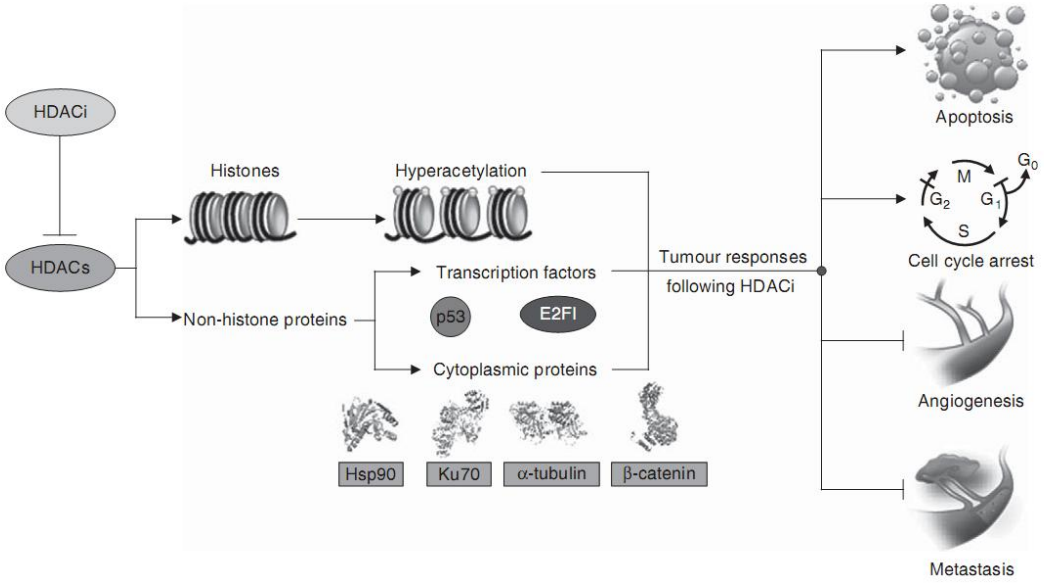

Figure 1.2 Anticancer effects of histone deacetylase inhibitors (HDACi) (149).

1.6.4.1 HDAC inhibitors in apoptosis

Inhibitors of HDACs have been found to have pro-apoptotic properties through either intrinsic or extrinsic apoptosis. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), death receptor 4 and 5 (Dc4 and Dc5), along with Fas-associated death domain (FADD) initiate extrinsic apoptosis downstream by activating caspase 8 and caspase 10. Activation of caspase 8 and caspase 10 cleave pro-caspase 3 and Bid, which engages the intrinsic apoptotic pathway in the mitochondria. The involvement of TRAIL associated with HDAC inhibitor, SAHA, results in cell death by activation of the caspase cascade and induction of mitochondrial-mediated cascade (150). HDAC inhibitors and TRAIL synergize to induce caspase 3 activation, internucleosomal DNA fragmentation, and promote mitochondrial damage (151). HDAC inhibitors, sodium butyrate and TSA, had similar effects on prostate cancer cells. Inhibition of HDACs decreased protein kinase casein kinase (PKCK) 2 activity, leading to caspase 2 activation and partial cleavage of caspase 8, sensitizing tumor cells to TRAIL-mediated cell death (152). Most of the studies investigated show that HDAC inhibitors are associated with TRAIL to synergize extrinsic and intrinsic apoptosis.

1.6.4.2 HDAC inhibitors in cell proliferation

Cell growth is tightly regulated via cell cycle, and growth is attenuated or terminated as a result of cell cycle arrest. The cell cycle can be blocked by cell cycle inhibitors, such as the *cip/kip* family and the INK4a/ARF (*In*hibitor of *K*inase 4/*A*lternative *R*eading *F*rame) family, each of which is involved in regulating different phases of cell cycle. HDAC inhibitors have been shown to increase the levels of $p21^{WAF1/Cip1}$ and $p27^{WAF1/Kip1}$ in vitro (153-157) and in vivo (158-159). Induction of p21 is tightly linked cell cycle arrest mediated by HDAC inhibitors in a

variety cell types through a p53-independent mechanism. This involves Sp1 binding sites in the p21 promoter region (159-160). In G1/S phase, retinoblastoma (Rb) forms a repressor containing HDAC and the hSWI/SNF, a member of E2F family. This complex inhibits transcription of genes for cyclins E and A (161) while cyclins E and A are suppressed by $p21^{WAF1/Cip1}$ and $p16^{INK4a}$. $p21^{WAF1/Cip1}$ and $p16^{INK4a}$ are both induced by HDAC inhibitors (162). In addition, $p21^{WAF1/Cip1}$ inhibits the association of phospho-Rb and E2F, thereby reducing the activity of E2F on cyclin E and CDK2, resulting in G1/S arrest.

1.6.4.3 HDAC inhibitors in angiogenesis and metastasis

The potential ability of HDAC inhibitors on tumor cell migration and invasion have been validated *in vitro*. Cell adhesion molecules such as E-cadherin, β-catenin and integrins have been reported to be regulated by HDAC inhibitors. TSA up-regulates integrins a4, b2 and b6 in Hep3B cells, inhibiting cell migration (163). HDAC inhibitors markedly induced growth response gene-1 (Egr-1), a Cys2-His2-typed zinc-finger transcription factor, and restored tight junctions (TJs) in cancer cells. These results suggest that HDAC inhibitors increase cell-cell adhesion. In addition, proteins that negatively regulate metastasis, such as thrombospondin- 1 (TSP-1), claudin-3 and E-cadherin were up regulated by HDAC inhibition and proteins that positively regulate metastasis, such as IGF-1R and snail (an EMT promoter), were reduced in human hepatocarcinoma cells (164). HIF-1α and VEGF were suppressed by TSA through an inhibition of the PI3K-AKT/PKC/HDAC pathway (165). Inhibition of these angiogenic factors inhibits endothelial cell migration, proliferation and angiogenesis (166-167). The same events have been demonstrated in other models by using bovine aortic endothelial cells and chick embryo (168). HDAC inhibitors such as FK228 (romidepsin), isolated from *Chromobacterium*

violaceum, blocked hypoxia-induced proliferation, invasion, migration, adhesion, tube formation and neovascularization (168).

1.6.4.4 HDAC and HDAC inhibitors in prostate cancer

Androgen receptor is an important transcription factor for cell growth and cell survival whether in hormone-sensitive or in castrated-resistant prostate cancer. There is increasing evidence demonstrating that AR is regulated by HDAC inhibitors. The effects of HDAC inhibitors have been determined in AR-dependent and AR-independent prostate cancer cell lines such as LNCaP, PC3 and CWR22Rv1 cells either *in vitro* or *in vivo* models. Valproic acid, an HDAC inhibitor that has been used clinically, reduced LNCaP cell proliferation by 14% when provided at physiological doses (0.45 mM) and abolished the growth promoting response of androgens by reducing AR expression (169). Similarly, valproic acid reduced PC3 cells proliferation by 40%, and the effects in both cell lines were associated with an increase of E-Cad (169).

Inhibition of HDACs with a variety of inhibitors or through siRNA technology suppresses transcriptional activity of the AR by inhibiting the assembly of co-activator/RNA polymerase II complex to target genes. In addition, HDAC inhibition can also reduce AR protein levels (170). In a CWR22Rv1 castrate-resistant prostate cancer xenograft model, LBH589 blocked tumor growth by 75% compared to control and reduced PSA gene expression (170). In contrast, Lee et al. reported that extracts of allspice reduced LNCaP cell growth by reducing histone acetyltransferase and histone acetylation through the inhibition of CBP/p300 (a protein complex with histone acetyltransferase activity). This reduction in histone acetyltransferase activity was accompanied by reduction in PSA gene expression, and a reduction of AR protein

expression (171). Collectively, it is suggested that high activity of HDACs causes epigenetic alterations (reductions in histone acetylation) and these changes are associated with an induction of malignancy.

There are two important features of HDAC inhibitors for use in the clinical setting. HDAC inhibitors are cytotoxic against tumor cells, but normal cells appear to be unaffected; and HDAC inhibitors have multiple activities affecting multiple molecular pathways. For example, they increase p21 expression via epigenetic regulation, and p53 through non-histone protein acetylation. Overall, HDAC inhibitors repress cancer cell proliferation, cell survival, metastasis and angiogenesis and induce cell apoptosis through transcriptional and post-translational modulation (172).

Despite what we know about HDAC inhibitors, the functions of HDAC isoforms in human tumors are not clear. Analysis of 192 samples of prostate carcinomas, revealed strong expression patterns of the class I family of HDACs, where 70%, 74% and 95% of the tumor samples had strong signals from HDAC1, HDAC2 and HDAC3, respectively. The expression of these HDACs was associated with tumor cell proliferation, and HDAC1 and HDAC2 expressions were significantly correlated with Gleason score. High expressions of HDAC1, 2 and 3 were accompanied by an earlier PSA-relapse after radical prostatectomy, showing an increase castrate-resistance (173). Therefore, HDACs might be a suitable target for treatment in prostate cancer. FK228 (romidepsin) has been approved by US Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL) and it has been used in a phase II clinical trial of progressing, metastatic, castration-resistant prostate cancer (CRPC), although there was only minimal antitumor activity in these patients (174). However, the combination of

HDAC inhibitor (vorinostat) and chemotherapy (doxorubicin) showed an antitumor effect without additional toxicity associated with chemotherapy alone (175). The combination of HDAC inhibitor SAHA (139) and radiation has been shown to enhance radiation-induced cytotoxicity in DU145 cells (androgen-independent prostate cancer cell line) (176), and this result provides a rationale for a combination therapy in further clinical trials.

1.7 Dietary components, phytochemicals, and prostate cancer

1.7.1 Anti-cancer properties of phytochemicals

Phytochemicals are non-nutritive components typically found in plants that have been shown to have multiple anti-carcinogentic and anti-mutagenic properties. These compounds are commonly used in the prevention and treatment of cancer/tumorigenesis using experimental models *in vitro* and in vivo, and in human clinical trials. The mechanisms of action of these phytochemicals involve multiple signaling pathways including suppression of PI3 kinase and MAP kinase pathways and inactivation of transcriptional factor such as nuclear factor k B (NFkB), resulting in cell cycle arrest and apoptosis.

1.7.1.1 Epidemiological and clinical evidences for an association between phytochemicals and prostate cancer

Prostate cancer is currently the most commonly diagnosed solid malignancy and has become the second leading cause of cancer-related deaths in men in most Western developed countries (3) and the use of phytochemicals in prostate cancer might be one of the more promising treatments. There is increasing evidence demonstrating dietary components, such as phytochemicals, may have utility in reducing the incidence of prostate cancer. Dietary

vegetables, fruits and medicinal herbs are rich in phytochemicals. Some studies indicated that the increased intakes of vegetables and fruits can lower the incidence and/or progression of prostate cancer. In an earlier multi-ethnic case-control study involving African-Americans, whites, Japanese, and Chinese from the United States and Canada, intakes of legumes, yellow-orange and cruciferous vegetables were inversely related to prostate cancer risk, but not tomatoes or fruits (177). Carotinoids, including lycopene-rich vegetables, reduced incidence and advanced forms of prostate cancer in a prospective cohort study and these effects were also observed in a phase II clinical trial (178-179). Allium vegetables, including garlic, scallions, onions, chives and leaks also decreased the risk of prostate cancer (180). In addition to vegetables and fruits, consumptions of food containing soy and isoflavones reduced the risk of cancer in a case-control study involving Chinese (181).

In addition to foods, common and medicinal herbs, including green tea, turmeric, etc., have been shown to have beneficial effects on reducing prostate cancer progression and incidence. Green tea has been used in China for thousands of years and green tea and its bioactive components reduce prostate cancer progression in different phases of the disease. In a proof-of-principle clinical trial, sixty individuals with high-grade prostate intraepithelial neoplasia (HGPIN) were randomly treated with green tea catechins (600 mg/day) or a placebo for a year. Only one individual was diagnosed with a malignancy in the group receiving catechins, while nine individuals in the placebo group developed prostate cancer (182). In a short-term study involving twenty-six men with positive prostate biopsies who were administered tea-derived polyphenols (1.3 g/day) , a majority of subjects had significant reductions in serum levels of hepatocyte growth factor (HGF), VEGF, IGF-I/ IGFBP-3 ratio, and PSA (183). These results indicated that the medicinal herbs or phytochemicals may have

anticancer properties.

1.7.1.2 The anti-carcinogenetic mechanisms of phytochemicals

Carcinogenesis is a multiple step process involving tumor initiation, promotion, and progression. Phytochemicals that are present in plants sources have been reported to possess anti-carcinogenic activities through multiple pathways (Table 2.1). Basically, these phytochemicals are classified into two categories: blocking agents and suppressing agents. Blocking agents prevent carcinogen formation from precursors and prevent carcinogens from reacting in the tissues. Suppressing agents inhibit, delay or reverse the expression of malignancy after exposure to carcinogens (184-185). Some phytochemicals inhibit pro-carcinogenic activity to electrophilic species or interaction with DNA and stimulate the detoxification of carcinogens. Other phytochemicals suppress the latter steps of cancer (promotion and progression) and some phytochemicals can act as both blocking and suppressing agents (186).

Phytochemicals, such as epigallocatechin-3-gallate (EGCG) and curcumin, have been shown to coordinately regulate the expression of xenobiotic metabolizing enzyme genes (phase I, II and III) and antioxidative stress genes (187-189), preventing tumor initiation by reducing pro-carcinogens, reactive intermediates or their activated metabolites and enhancing the cellular defense system (187-188). In addition, phytochemicals have been shown to generate reactive oxygen species (ROS) to disrupt the mitochondrial membrane potential and activate the intrinsic apoptotic cascade. Phytochemicals, such as berberine, can increase the production of ROS by increasing xanthine oxides in prostate cancer cells (12).

Phytochemicals also act as suppressing agents and function on tumor promotion and progression by coordinately regulating various signaling pathways, including growth factor receptors pathways, stress-activated protein kinases pathways, nuclear factors signaling and the regulation of the cell cycle and cell death.

1.7.1.2.1 Modulation of growth-factor receptors pathways by phytochemicals

Increasing evidence indicates growth factors and/or growth factor receptors are over expressed in tumor cells and contribute to cell proliferation and survival signals. Phytochemicals have been shown to reduce growth factor expression and down regulate the expression and activity of growth factor receptors. Curcumin was the first phytochemical found to inhibit tyrosine phosphorylation of epidermal growth factor receptor (EGFR) (190-191) and human epidermal growth factor receptor 2 (Her2/neu) (192). It was also found that curcumin sensitized prostate cancer cells to apoptosis induced by TRAIL by activating c-Jun N-terminal kinase (JNK), inhibiting IκBα/NFκB activation, and suppressing AKT signaling pathways (193-196), resulting in activation of the caspase cascade, and thus cell death (197). The ability of curcumin to sensitize cells to TRAIL was demonstrated in a xenograft model using TRAIL-resistant LNCaP prostate cancer cells. Mice were provided TRAIL (15 mg/kg) in the presence or absence of curcumin (30 mg/kg body weight). Compared to TRAIL alone, curcumin sensitized LNCaP cells and by reducing VEGF, VEGFR2, NFκB-induced COX2, MMP2 and MMP9 while inducing death receptor 4 and 5, p21 and p27 (198).

Additionally, the proliferative EGFR-dependent Erk1/2 signaling pathway can be inhibited by EGCG (199). EGCG also inhibited IGF-1-induced cell growth in androgen-dependent and androgen-independent prostate cancer cells (200-201) and inactivated

VEGF/VEGFR axis by suppressing the expression of hypoxia-inducible factor (HIF)-1α, reducing angiogenesis (202). The reductions in growth-factor receptors pathways were also observed in other phytochemicals such as resveratrol (15), gingerol (203), baicalein (14), berberine (204).

1.7.1.2.2 Modulation of stress-activated pathways by phytochemicals

Extracellular stresses activate several pathways including stress-activated protein kinases and p53 expression. Stress-activated protein kinases include JNKs and p38 mitogen-activated protein kinase (MAPK). They are activated in response to the extracellular stimuli, such as UV, DNA-damage agents or pro-inflammatory factors. These kinases function as either pro-apoptotic protein kinases or anti-apoptotic kinases (205). Curcumin has been shown to have multiple anticancer properties including induction of JNK and p38 MAPK, cytochrome c release from mitochondria and activation of caspases 3, 8 and 9 in androgen-independent PC3 prostate cancer cells (10). Similar results were observed when PC3 cell were treated with a variety of flavonoids (kaempferol, apigenin, genistein and naringenin). Inductions in phospho-JNK, phospho-Erk and increases in activator protein 1 (AP-1), a downstream transcription factor of JNK, was observed in PC3 cells (206). Transcription factor AP-1 plays an important role in carcinogenesis and regulates a variety of its target genes, such as EGFR, cyclin D1, Bcl-2 and MMPs (207-210). In androgen-dependent LNCaP prostate cancer cells, protoapigenone, a natural derivative of the flavanoid apigenin, showed similar effects on JNK and p38 MAPK activations (211). These studies indicate that the treatments of flavonoids can result in activation of JNK and in cell death.

In addition to AP-1 activation through JNK or p38 MAPK signaling, NFκB is a rapid-activated stress-responsive transcription factor, inducing the transcription of various genes encoding cytokines, membrane proteins, other transcription factor, inhibitors of apoptosis and pro-inflammatory factors (212). NFκB activation is initiated through a removal of inhibitory protein (IκB) from the NFκB/IκB complex by a variety of stimuli including TNFα, LPS, UV or IR and DNA damage (213). In cervical cancer cells, curcumin reduced TNFα-induced NFκB activation and reduced cyclooxygenase 2 (COX-2) expression through a blockage of IκBα phosphorylation, increasing degradation (214). In addition, Curcumin has anti-inflammatory property by reducing leukocytes recruitment mediated by TNFα-induced NFκB activation (215). Resveratrol has also been shown to reduce NFκB expression by targeting protein NAD(P)H dehydrogenase and quinone 2 (NQO2) in CWR22Rv1 prostate cancer cells (216). Resveratrol also inhibited TNFα-induced Erk and JNK activation and reduced p65 phosphorylation and nuclear translocation (217). Other phytochemicals including EGCG (218), genistein (219), [6]-gingerol (11), capsaicin (220) and caffeic acid phenethyl ester (CAPE) (221) have the similar inhibitory effects on NFκB activation induced by UV light, TNFα, 12-O-tetradecanoylphorbol-13-acetate (TPA) or phorbol 12-myristate 13-acetate (PMA).

Tumor suppressor gene, p53, inhibits tumorigenesis in responding to cellular genotoxic stresses, such as UV, IR or non-genotoxic stress, such as phytochemicals (222). Activation of p53 induces cell cycle arrest and cell apoptosis resulting in reducing cancer cell growth. Lack of p53 is associated with an increase of tumorigenesis, allowing an accumulation of mutations in damaged cells and blocking the apoptotic responding to genotoxic stress. Phytochemicals, EGCG, genistein, quercetin, indole-3-carbinol, curcumin and resveratrol, have been shown to increase p53 expression, induce cell cycle arrest and cell death in both androgen-dependent

LNCaP and androgen-independent PC3, DU145 and CWR22Rv1 prostate cancer cells (223-228). Activation and induction of p53 by phytochemicals such as resveratrol and caffeine are regulated by MAP kinases on p53 phosphorylation at serine 15, which stabilize and activate p53 (229-231). Resveratrol inhibits the growth of LNCaP prostate cancer cell by activating p53-responsive genes such as $p21^{WAF1/CIP1}$, p300/CBP and Apaf-1 (232). Additionally, in LNCaP cells, cell apoptosis is promoted and the cell growth is inhibited by EGCG treatment along with induction of p53, reduction of NFκB, and suppression of Bcl-2 (233). Activation and phosphorylation of p53 can be induced through ataxia teleangetasia mutated and ATM-related kinase (ATM/ATR) pathway by genistein and enhances the sequence-binding specificity of p53 on target DNA (228). While p53 is up-regulated and activated by phytochemicals, activated p53 promotes cell cycle arrest and cell death, thus inhibiting tumorigenesis.

1.7.1.2.3 Induction of cell cycle arrest and apoptosis by phytochemicals

The majority of phytochemicals reduce tumor cell proliferation by inducing cell cycle arrest and increasing cell death. Cell cycle mediators and cell death promoters are modulated by phytochemical in a variety of cancer cell lines (234). Resveratrol is one of the more widely studied phytochemicals. When LNCaP, DU-145, PC-3, and JCA-1 human prostate cancer cells were treated with resveratrol, G1/S transition phase of the cell cycle was inhibited and cell apoptosis was induced (235). The inhibition effects of resveratrol on cancer cell growth have been found in other cells including breast, colon, pancreas, and squamous cell carcinoma etc. Resveratrol increased cell cycle inhibitors, p21 and p27, and tumor suppressor gene, p53, along with reductions in cell cycle promoter, cyclin D1 and cyclin E, anti-apoptotic proteins, Bcl-2 and Bcl-xL. Proliferative AKT signaling was also decreased by resveratrol (236). p21 and p27 inhibit

cell cycle regulators, cyclin/ cyclin-dependent kinase, thus blocking cell cycle transition. In addition, it has been shown that resveratrol enhances apoptosis induced by TRAIL through induction of cellular inhibitor-of-apoptosis proteins (cIAPs) and Bax expression (237, 13, 238), and reduction of AKT phosphorylation (239) in androgen-dependent and androgen-independent prostate cancer cells.

In animal models, green tea and its bioactive compounds, catechins, have been shown to inhibit tumor cell growth. In a CWR22Rv1 athymic nude mouse model, green tea polyphenols (GTP) and EGCG attenuated tumor growth by extending the length of time to reach final tumor size by 38% and 108%, respectively. Reduced Bcl-2 expression and increases in active caspase 3, cleaved PARP and Bax expression indicated EGCG and GTP inhibited prostate cancer cell growth by increasing apoptosis (240). The mechanisms of EGCG on prostate cancer cell growth have been investigated using PC3 and LNCaP cells. EGCG induces cell cycle arrest by increasing p53-dependent p21 expression and promotes cell death by reducing pro-proliferative Bax expression and NFkB transcriptional activity (241, 233), thereby inhibiting cell growth.

Table 1.3 Summary of molecular targets by curcumin and EGCG in prostate cancer animal and cell models

phytochemicals	doses	Molecular targets	Animal models/ cell types	Ref.
Curcumin	5-50 μM	↓ EGFR	LNCaP; PC3	(190)
	10 μM	No changes on Her2/neu, PARP, STAT3 DNA binding activity	PC3 , DU145	(242)
	10-50 μM	↓ IκBα/NFκB; ↓ p-AKT, Bcl-2, Bcl-xL, XIAP; ↑ caspase	LNCaP, PC3	(193-194)
	25-100 μM	↑p-p38, p-JNK, caspase 3, 8, 9, Cyt C, AIF	PC3	(10)
	5-30 μM	↓p-AKT ↓Bcl-2, Bcl-X ↑ROS ↑p53, Bax, Bak, PUMA, Noxa, Bim, Cyt c, Smac/DIABLO, Omi/HtrA2, caspase 3; ↑Acetylation of histone H3, H4	LNCaP	(227)
	30 mg/kg	↓ VEGF, VEGFR-2, MMP2, MMP9, COX2, p65; ↓ cyclin D1, Bcl-2, Ki67; ↑ DR4,DR5, Bax; ↑ $p21^{WAF-1}$, $p27^{KIP-1}$	LNCaP in Athymic (nu/nu) nude mice	(198)

Table 1.3 Continued.

phytochemicals	doses	Molecular targets	Animal models/ cell types	Ref.
EGCG	5-20 μM	↓ IGF1, AKT,	LNCaP, DU145, PrEC	(200-201)
	2.5 μM	↓AR; ↑p53	CWR22v1	(226)
	5-40 μg	↓ cyclin D1, cyclin E, cdk2, cdk4, cdk6; ↑ $p16^{INK4a}$,$p18^{INK4c}$, $p21^{WAF-1}$, $p27^{KIP-1}$	LNCaP, DU145	(225)
	20-80 μM	↓ NFκB; ↑p53, $p21^{WAF-1}$, Bax, caspase 3, 8, 9	LNCaP	(241, 233)
	1 mg/ day	↓ PSA, tumor size; ↓ VEGF, Bcl-2; ↑ caspase3, PARP, Bax	CWR22Rnu1 in Athymic (nu/nu) nude mice	(240)

1.7.1.3 Epigenetic anti-cancer effects of phytochemicals

Phytochemicals have been shown to regulate gene expression epigenetically via DNA methylation and histone acetylation and methylation. Methylation on CpG site in DNA by DNA methyltransferases (DNMTs) is an epigenetic silencing mechanism on tumor suppressor genes during tumorigenesis, and removal of methyl groups from the CpG islands allows binding of transcriptional proteins. This removal can be induced by phytochemicals such as EGCG, curcumin and others. In addition, histone modification is another key feature of epigenetic regulation through acetylation and methylation. These modifications on histones allow the loosing of the chromatin structure resulting in the recruitment of transcriptional factors on DNA, thus increasing gene expression. A number of phytochemicals have been shown to modify histones, enhancing the expression of tumor suppressor genes, such as p21. It has been demonstrated that the epigenetic regulation by diet is inherited in mice (243), indicating that dietary factors are able to influence gene expression.

1.7.1.3.1 DNA methylation

One of the epigenetic changes in prostate cancer is DNA methylation. More than 30 genes have been identified to have abnormal methylation on DNA promoters including the genes related to the cell cycle, hormone response, and DNA repair (244). For example, gluthathione-*S*-transferase pi (GSTP1) is involved in detoxification and protects DNA from reactive electrophilic intermediates. Hypermethylation results in gene silencing and this phenomenon has been found in LNCaP, C4–2B, PC-3, DU-145, LAPC-4, and VCaP human prostate cancer cells. This observation is supported in metastatic prostate cancer tissues compared to normal tissue (245). Green tea polyphenols (GTPs) and its bioactive compound, EGCG, are reported to re-

expression of GSTP1 (246). DNA methyltransferase 1 functions by adding methyl groups on the DNA promoter region, resulting in DNA silencing. The activity of DNMT1 in LNCaP, MDA, PCa 2b and DU145 human prostate cancer cell lines were suppressed by EGCG (247). EGCG binds to catalytic residues on DNMT1 through hydrogen bonding and blocks the active site of DNMT1, reducing DNMT1 activity (248). In addition to the inhibitory effect of EGCG on DNMT1 kinetic activity, the DNMT1 inhibitor, *S*-adenosyl-$_L$-homocysteine (SAH), is increased by EGCG by an enzymatic methylation reaction (249). These results explain the epigenetic regulation mechanisms of EGCG *in vitro*. However, the underling mechanisms of EGCG via mediation of DNMTs on tumor suppressor genes have not been illustrated in prostate cancer patients yet.

1.7.1.3.2 Histones acetylation and methylation

Histone acetylation and methylation are the principle regulators of epigenetic regulation of gene expression involving histones. The actions of acetylation and methylation on specific lysine or arginine residues in histones H3 and H4 are reversible processes that are modulated by histone acetyltransferases (81), HDACs and histone methyltransferases (HMTs) (250). Histone deacetylases are the new target for cancer chemotherapy, including prostate cancer, with elevated expressions of HDAC1, 2 and 3 (173). The use of HDAC inhibitors, such as TSA, has been shown to inhibit HDAC expression and activity and induce the cell cycle inhibitor, p21. This results in cell cycle arrest and cell death (using *in vitro* models) (160), and thus HDACs inhibitors are a therapeutic target for the inhibition of tumor progression. Important to this discussion is the fact that phytochemicals appear to act as HDAC inhibitors, arresting tumor cell growth and promoting cell death. Isothiocyanate is found in cruciferous vegetables, such as

broccoli, and has been shown to inhibit HDACs *in vitro, in vivo* and in tissues from human subjects (251). For example, consumption of broccoli sprouts (68 g), containing 105 mg of the isothiocyanate sulforaphane, reduced plasma HDAC activity in human subjects after 3 hours (252). In a xenograph mouse model using PC3 human prostate cancer cells, sulforaphane (443 mg/kg diet for 21 days) reduced tumor growth, inhibited HDAC activity, and increased acetylation of histones H3 and H4 (252).

Regulation of histone acetylation is a balance between the removal and incorporation of acetyl groups mediated by HDACs and histone acetyltransferases. Induction of histone acetyltransferases counter balances the actions of HDACs. Ideally, if hyperacetylation of histones were the overall goal, then it would be most desirable to use a product that inhibits HDAC function and enhances histone acetyltransferase activity. The classical HDAC inhibitor TSA has been shown to increase acetylation of CBP/p300 and thus increase its acetyltransferase activity (253-254). The mechanism of activation is through MAP kinase signaling (255). Curcumin has been shown to increase this pathway via MAP kinase phoshporylation with concomitant induction of p21 expression, negatively regulating the cell cycle (256). This effect of curcumin to inhibit tumor cell growth is supported by similar results in LNCaP prostate cancer cells. Curcumin inhibited ntunor growth and induced the acetylation of histones H3 and H4 (227).

In addition to histone acetylation, methylation on specific lysine or arginine residues in histones H3 and H4 by HMTs is another process either to activate or to repress gene expression. When compared to post-treatment (radiotherapy + hormone therapy) plasma levels of circulating histone H3 lysine 27 trimethylation (H3K27me3) from organ confined (n=22), locally advanced (n=11), and metastatic (n=28) prostate cancer patients, the median level of plasma H3K27me3 in

the patients with metastatic PCa was lower than patients with localized or locally advanced prostate cancer (257), indicating that histone methylation is associated with progression of the disease. This result is consistent with a finding that JMJD3 (defined as a histone demethylase) is upregulated in advanced and metastatic prostate cancer patients (258). JMJD3 and H3K27me3 might be used as biomarkers of advanced of prostate cancer.

1.7.2 The use of phytochemicals in combination on prostate cancer

Consumption of whole fruits, vegetables and whole grain is strongly associated with a reduction of prostate cancer risk (180, 177, 259). The effective bioactive compounds in the fruits, vegetables and whole grains have been identified. The molecular mechanisms of these bioactive compounds on tumorigenesis are well studied using experimental models in vitro and in vivo (260, 198, 240, 185). However, it is believed that single nutrients or phytochemicals when used at physiological concentrations, do not have the same effects as the whole fruits, vegetables and whole grains as reported in epidemiological studies. In a 12- year randomized, double-blind, placebo-controlled trial of beta-carotene (50 mg/d on alternate days), Hennekens et al. found that the participants receiving beta-carotene had significantly higher beta-carotene concentrations in plasma than those given placebo, but there were no significant differences in the number of cases of lung cancer, or with other cancers, including colon/rectum, prostate , stomach, pancreas, brain, or with various types of cancers, such as melanoma, leukemia, or lymphoma (261). These results suggest that a single compound might as bioactive against tumorigenesis as compared to the food it is found in. One of the possible strategies is the use of combinations of bioactive compounds, whereby combinations may act synergistically to inhibit tumor growth. For example, when tea polyphenols were provided to men with positive prostate biopsies, there was a significant

reduction in serum PSA levels, but this effect was not observed with isolated catechins (183). In a CWR22Rv1 athymic nude mouse model, green tea polyphenols attenuated tumor growth compared to mice receiving one of its bioactive components, EGCG (240). A combination of soy phytochemicals (5 g/kg diet) and tea (15 g tea leave/L drinking water) for 10 weeks showed a synergistic inhibition on prostate tumor growth, metastasis and angiogenesis in LNCaP prostate cancer xenograft mice (262). The same effects have been observed in other cancers, such as lung and colon in mouse models (263-264). These synergistic effects of a combination of phytochemicals may explain the efficacy of fruits and vegetables.

1.7.2.1 An example of the use of phytochemicals in combination, Zyflamend®

Zyflamend® is a multi-herb extract with anticancer properties. Extracts from each herb contain a variety of bioactive phytochemicals (i.e., berberine, curcumin, epigallocatechin gallate, resveratrol, etc), with known efficacy. The individual chemistries have been shown to increase cell death and suppress cell proliferation via a variety of mechanisms (10, 265, 12, 203, 202, 14-15). Therefore, their unique combination could result in additive or synergistic effects at relatively low concentration. Zyflamend® appears to provide clinical improvement to patients diagnosed with HGPIN, an early preneoplastic form of the disease. In a phase I clinical study, it was reported that 48% of HGPIN patients had a 25-50% reduction in PSA levels and a reduction in NFκB expression after a 18-month Zyflamend® (2.3g/d) (266-267). Using In vitro studies, Zyflamend® reduced the growth of androgen-dependent LNCaP cells and this reduction was associated with an elevated expression of p21 and apoptosis (as indicated an induction of PARP cleavage and caspase 3 activity). Androgen receptor expression was also reduced by Zyflamend® (268). In PC3 cells, Zyflamend® reduced Rb phosphorylation and thus blocked

G2/M phase in the cell cycle. The reduction of Rb was related to inhibition of 12-lipoxygenase (269). These anticancer properties of Zyflamend® were also observed in other cell models, including human non-small cell lung carcinoma (H1299), human myelogenous leukemia (KBM-5), and human multiple myeloma (U266) cells (suppressing osteoclastogenesis). Zyflamend® induced cell cycle arrest and apoptotic and anti-angiogenetic gene expression by repressing NFκB and its downstream genes, such as Bcl-2, Bcl-xL, FADD-like interleukin-1β converting enzyme /caspase-8 inhibitory protein (270). However, the anticarcinogenic properties of Zyflamend® on various stages of advance prostate cancer stages and the progression of the disease from androgen-dependent to castrate-resistant growth in conjunction with hormone deprivation has yet to be explored. Because of the diversity of chemo-preventive phytonutrients in Zyflamend®, the use of Zyflamend® as an adjuvant to standard therapies, such as hormone ablation therapy, seems promising.

1.8 Summary

Prostate cancer is a complicated disease involving multiple mechanisms and using a therapy that has a specific target may not be as effective as a strategy that takes advantage of the complexity associated with foods and herbs, thereby simultaneously affecting many signaling pathways.

In this study, we decided to use a mixture of the extracts of ten individual herbs, Zyflamend®, many of which having anticancer properties. We chose only to use human equivalent does when provided in a diet, and concentrations we believed to be physiologically relevant (in vitro). This was important to insure some translatability. We chose a human prostate cancer cell line model, CWR22, that shared important characteristics related to the progression

of human prostate cancer. We would like to discover if this mixture can reduce prostate cancer growth and delay progression in a model of advance prostate cancer in the presence or absence of androgens.

1. Determine the effects of Zyflamend® on androgen-dependent tumor growth in vivo.
2. Determine the effects of Zyflamend® on tumor regression in conjunction with hormone deprivation in vivo.
3. Determine the effects of Zyflamend® on castrate resistant tumor growth in vivo and in vitro.
4. Determine underlying molecular mechanisms associated with some of these effects.

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Chapter II

Epigenetic Regulation of p21 and Chemopreventive Effects of a Combination of Herbal Extracts in Castrate-Resistant Prostate Cancer

Abstract

Zyflamend, a mixture containing extracts of ten common herbs, has been shown to cause regression of early stages of prostate cancer in clinical trials. The current experiments were designed to investigate its effects on advanced prostate cancer. Zyflamend® inhibited growth of human CWR22Rv1 prostate cancer cells by increasing the expression of the cell cycle regulators p21 and p27 through a p53-independent mechanism. Overexpression of p21 by Zyflamend® was associated with a suppression of class I and II histone deacetylases (HDACs) and an induction of CBP/p300 histone acetyl transferase activity by up regulating Erk-1/-2 and Elk-1. These effects were not observed with the individual herbal extracts. Our results suggest the extracts of this herbal combination inhibits castrate-resistant prostate cancer cell growth epigenetically by coordinately affecting multiple signaling pathways involved in histone acetylation.

2.1 Introduction

Prostate cancer (PrC) is currently the most commonly diagnosed solid malignancy and has become the second leading cause of cancer-related deaths in men in most Western developed countries (1). Metastatic PrC is defined as the spread of PrC cells to secondary sites (i.e., bone, lung, etc). Once tumors become metastatic, they are very difficult to treat, and prognosis is poor with a 31% 5-year survival rate (2). For the most part, PrC is temporarily responsive to hormone deprivation therapy as prostate epithelial cells are dependent on androgens for growth. While treatment with hormone deprivation results in tumor regression and clinical stabilization, the disease eventually relapses, with invariable fatal results within two years. Therefore, a critical barrier in treating advanced PrC is finding effective treatments for castrate-resistant forms of the disease. The CWR22Rv1 PrC cell line was chosen for the experiments because it represents a very late stage of PrC (3). These cells can grow in the presence or absence of androgens, produce prostate specific antigen (PSA) and express a functional androgen receptor (3) and, as such, these cells are considered to be a castrate-resistant cell line. Similarly, PrC in patients whose disease has relapsed following androgen ablation therapy expresses androgen receptors and produces PSA.

The use of herbs, botanicals and their bioactive components have been shown to be effective in many tumor cells lines *in vitro* and *in vivo* by inhibiting cell and tumor growth. The use of herbal extracts in combination potentiates their actions, resulting in significant activity when any single agent has no effect (4-7). Zyflamend®, a polyherbal preparation derived from the extracts of ten common herbs, appears to provide clinical improvement to patients diagnosed with high grade prostatic intraepithelial neoplasia (HGPIN), a preneoplastic form of the disease

(8). In addition, Zyflamend® has been shown to reduce proliferation in a variety of PrC cell lines by modulating genes that impact the cell cycle and apoptosis (9-11). Of particular interest to our laboratory is the effect of Zyflamend® on castrate-resistant PrC.

In this study, we investigated the epigenetic effects of Zyflamend® on expression of the cell cycle regulator p21 and concomitant inhibition of cell proliferation. Expression of p21 is regulated, in part, by histone acetylation as mediated by histone deacetylases and histone acetyl transferases. This work was designed to explore some of the molecular mechanisms behind epigenetic regulation and the anti-carcinogenic effects of Zyflamend®.

2.2 Materials and Methods

2.2.1 Zyflamend®

Zyflamend® (New Chapter, Brattleboro, VT) is derived from the extracts of ten different commonly consumed herbs (w/w): holy basil (12.8%), turmeric (14.1%), ginger (12.8%), green tea (12.8%), rosemary (19.2%), hu zhang (10.2%), barberry (5.1%), oregano (5.1%), baikal skullcap (2.5%), and Chinese goldthread (5.1%).

2.2.2 Cell Culture

Human prostate cell lines, RWPE-1, LNCaP, PC3 and CWR22Rv1, were purchased from American Type Culture Collection (Rockville, MD). RWPE-1 cells were maintained in complete medium containing keratinocyte serum free medium supplemented with bovine pituitary extract (BPE) (0.05 mg/ml) and human recombinant epidermal growth factor (hEGF) (5 ng/ml). LNCaP, PC3 and CWR22Rv1 cells were maintained in RPMI 1640 media supplemented with 10% fetal

bovine serum (FBS) under an atmosphere of 5% CO2 at 37oC. Cells were harvested with the addition of 0.25% trypsin with 0.02% EDTA during the exponential growth phase. For the experimental treatments, cells were cultured in RPMI 1640 media supplemented with 0.05% fetal bovine serum containing Zyflamend® or individual herbal extracts (ginger, rosemary, turmeric, Chinese goldthread, holy basil, hu zhang, barberry, green tea and baikal skullcap) (each supplied by New Chapter, Inc, Brattleboro, VT) reconstituted in dimethyl sulfoxide (DMSO) for cell proliferation assay, mRNA extraction and protein isolation. For inhibitor experiments, CWR22Rv1 cells were pretreated with U0126 (Erk inhibitor) (Cell Signaling Technology, Inc., Danvers, MA) at a dose of 2 μM for 30 minutes and subsequently treated with Zyflamend® (200 μg/mL) for 24 hr. For histone deacetylase (HDAC) experiments, trichostatin A (TSA) (a general HDAC inhibitor) was added alone at various concentrations (0, 0.1, 0.5, 1.0 μM) or in combination with Zyflamend® (200 μg/mL) for 24 hours. In all experiments, 0.1% DMSO was used as the vehicle control.

2.2.3 Cell Proliferation

The MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Andwin Scientific, Addison, IL) was used to assess relative cell growth and viability, following the manufacturer's instructions (Promega, Madison, WI). Cells (1×104 cells of RWPE-1, LNCaP, and CWR22Rv1, and 5×103 cells of PC3) were plated in 96-well plates in a volume of 100 μl culture medium. The culture medium contained various concentrations of Zyflamend® (0, 40, 80, 100, 150, 200 μg/mL) or individual herbal extracts (10.2 μg/ml ginger, 15.4 μg/ml rosemary, 11.3 μg/ml turmeric, 4.1 μg/ml Chinese goldthread, 10.2 μg/ml holy basil, 8.2 μg/ml hu zhang, 4.1 μg/ml barberry, 10.2 μg/ml green tea, 2.0 μg/ml baikal skullcap; equivalent to

those doses in 200 μg/ml Zyflamend®). Cell proliferation was determined at 0, 24, 48, 72, 96 hr post incubation. At each time point, a mixture of MTT:complete medium (1:10, v/v) was added and incubated at 37℃ for 4 hr in a CO2 incubator (5%). Absorbance (at 540 nm) was measured on a SpectraCount microplate photometer (Perkin Elmer Inc, Waltham, MA).

2.2.4 BrdU incorporation Assay

Cells (1×104 cells of CWR22Rv1) were plated in 96-well plates and treated with various concentrations (0, 100, 150 and 200 μg/ml) of Zyflamend® for 48 hr and followed by a BrdU incorporation assay to assess relative DNA synthesis following the manufacturer's instructions (EMD Biosciences, Inc., Darmstadt, Germany). After Zyflamend® treatment, cells were treated with BrdU for 4 hr and the BrdU incorporation was measured on a FluoroCount microplate photometer (Perkin Elmer Inc, Waltham, MA) at a 340 nm excitation and a 460 nm emission.

2.2.5 Down regulation of p21 by small interfering RNA

CWR22Rv1 (1.5×105 cells per well in 6-well plates, in serum-free RPMI 1640 media) were transfected with validated p21 small interfering RNA (siRNA) or Stealth™ siRNA negative control (Invitrogen, Carlsbad, CA) (100 pmole each) using Lipofectamine 2000™ transfection reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. Six hrs post transfection, cells were cultured with RPMI 1640 media containing 10% FBS overnight. After recovery, media was replaced with 0.05% FBS media containing vehicle or Zyflamend® (200 μg/ml) for 24 hr at 37 ℃. The total RNA was harvested for quantitative real-time polymerase chain reaction (qRT-PCR) and cell number was determined.

2.2.6 Overexpression of p21

pRc/CMV-p21 (Addgene Inc., Cambridge, MA), containing full length wild-type p21 cDNA, was used to overexpress p21. CWR22Rv1 cells (1.5×105 cells per well in 6-well plates) were plated overnight. pRc/CMV-p21 or pRc/CMV (empty vector) were transfected using Lipofectamine 2000™ reagent in serum-free RPMI 1640 media. Transfected cells were selected by treatment for two weeks with neomycin (50 μg/ml) and subjected to the MTT cell proliferation assay. p21 protein expression in the transfected cells was examined by western blot.

2.2.7 RNA isolation and quantitative RT-PCR

Total RNA was isolated from CWR22Rv1 cells using Trizol reagent (Invitrogen, Carlsbad, CA) followed by chloroform extraction. The aqueous phase was precipitated in 100% isopropanol and the pellet was washed in 75% ethanol prior to re-suspension in RNase-free water. Contaminating DNA was removed from RNA samples using Turbo DNA free™ kit (Ambion, Inc. Austin, TX) and then the concentration of total RNA was measured using NanoDrop 1000™ (Thermo Scientifc Wilmington, DE). Total RNA (2 μg) from each sample was mixed with MultiScribe™ Reverse Transcriptase, RNase Inhibitor, dNTP Mixture, random hexamers, RT buffer, MgCl2 solution and incubated at 25℃ for 10 min, 48℃ for 30 min and 95℃ for 5 min to reverse transcribe to cDNA using TaqMan® reagent kit (Applied Biosystems, Carlsbad, CA). cDNA samples were used for quantitative RT-PCR (ABI Prism® 7300 Real-Time PCR System, Applied Biosystems, Carlsbad, CA). cDNA (0.7μl) was used as a template for qPCR amplification with primer sets (2.5 μM) of p21 sense, 5’-TGGAGACTCTCAGGGTCGAAAA-3’ and antisense, 5’- CCGGCGTTTGGAGTGGTA-3’, p27 sense, 5’-CGGTGGACCACGAAGAGTTAA-3’ and antisense, 5’-GGCTCGCCTCTTCCATGTC-3’,

HDAC1 sense, 5'-ACCGGGCAACGTTACGAAT-3' and antisense, 5'-CTATCAAAGGACACGCCAAGTG-3', H DAC2 sense, 5'-TCATTGGAAAATTGACAGCATAGT-3' and antisense, 5'-CATGGTGATGGTGTTGAAGAAG-3', HDAC3 sense, 5'- TTGAGTTCTGCTCGCGTTACA-3' and antisense, 5'-CCCAGTTAATGGCAATATCACAGAT-3', HDA4 sense, 5'-AATCTGAACCACTGCATTTCCA-3' and antisense, 5'-GGTGGTTATAGGAGGTCGACACT-3', HDAC5 sense, 5'-TTGGAGACGTGGAGTACCTTACAG-'3 and antisense 5'-GACTAGGACCACATCAGGTGAGAAC-3', HDAC6 sense, 5'-TGGCTATTGCATGTTCAACCA-3' and antisense 5'-GTCGAAGGTGAACTGTGTTCCT-3', HDAC7 sense, 5'-CTGCATTGGAGGAATGAAGCT-3' and antisense 5'-CTGGCACAGCGGATGTTTG-3', were examined. Amplification was performed using a standard thermocycle program beginning with an initial temperature at 94°C for 1 min followed by 30 cycles of 94°C for 15 sec, 50°C for 30 sec and 72°C for 2 min. Each sample was examined in triplicate and the amounts of PCR product were normalized with 36B4, 5'-TGCATCAGTACCCCATTCTATCA-3' and 5'-AAGGTGTAATCCGTCTCCACAGA-3' as the internal control.

2.2.8 Protein degradation

CWR22Rv1 cells were cultured with RPMI 1640 medium containing Zyflamend® (200 μg/ml) for 24 hr to induce p21 expression. Cells were then cultured ±Zyflamend® for an additional 24 hr. In addition, cells were treated with Zyflamend® (200 μg/ml) for 24 hr prior to adding cycloheximide (10 μM) to terminate protein synthesis for an additional 0, 0.5, 1, 1.5, 2, 4

hr in the continued presence or absence of Zyflamend® (200 μg/ml) and harvested for protein analysis.

2.2.9 Western blotting

CWR22Rv1 cells were lysed in the presence of cell lysis buffer (Cell Signaling Technology®, Inc. Danver, MA). Protein content of the lysates was quantified by BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Lysates (20 μg) were fractioned by 8~12% SDS-PAGE and transferred to a polyvinylidine diflouride (PVDF) membrane by electroblotting. The membranes were blocked using 5% nonfat dry milk in 0.1% Tris-buffered saline-Tween-20 (TBST) for 1 hour at room temperature and incubated in TBST containing primary antibodies overnight at 4°C. The membrane was incubated with anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) (Cell Signaling Technology®, Inc. Danver, MA). Protein expression was detected with a Pierce ECL Western Blotting detection system. Each membrane was exposed to Hyperfilm Film (GE Healthcare, Piscataway, NJ). Antibodies of p21, p27, p53, cyclin D1, cyclin E, HDAC1-7, Erk, phospho-Erk, Elk-1 (Cell Signaling Technology®, Inc. Danver, MA), phosphor-Elk-1 (GenScript USA, Inc. Piscataway, NJ) were used. Actin (with anti-β-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)) was used as the control.

2.2.10 HDAC activity assay

CWR22Rv1 cells were lysed in the presence of cold lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 15 mM Mg2Cl2, 250 mM sucrose, 0.5% NP-40, and 0.1 mM EGTA). Nuclei pellets were separated through a sucrose cushion (30% sucrose, 10 mM Tris-HCl, pH 7.5, 10 mN

NaCl, and 3 mM Mg2Cl2) and resuspended in extraction buffer (50 mM HEPES, pH 7.5, 420 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, and 10% glycerol). The nuclei suspension was sonicated on ice for 30 seconds and then the supernatant was stored at -80°C. An aliquot (30 µg/10 µl) of the nuclear extracts was used for HDAC activity assay as per manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). The assay was measured using an excitation wavelength of 340 nm and an emission wavelength of 460 nm (FluoroCount, Perkin Elmer Inc, Waltham, MA).

2.2.11 Statistic analysis

The results are presented as mean ± SEM and the mRNA results are presented as mean ± SD. The results were analyzed by two-way ANOVA. Differences were considered significant at $p<0.05$ versus control.

2.3 Results

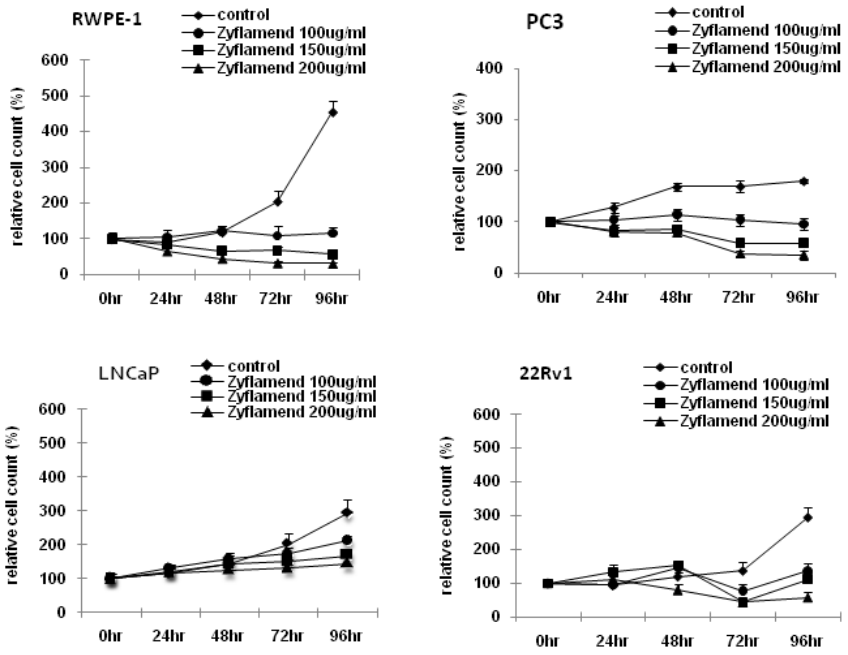
2.3.1 Prostate cancer cell growth and DNA synthesis were inhibited by Zyflamend®

Zyflamend® inhibited growth in all PrC cell lines tested in a time and concentration-dependent manner. At the end of 96 hr treatment, Zyflamend® inhibited cell growth in RWPE-1 cells by 80%, LNCaP cells by 60%, PC3 cells by 50% and CWR22Rv1 cells by 75% (Fig.2.1A). To further confirm the reduction of cell proliferation by Zyflamend®, BrdU assay was used for determining DNA synthesis during the cell cycle. After treatment with Zyflamend® (48 hr), BrdU incorporation in CWR22Rv1 cells was reduced in a concentration-dependent manner compared to control (Fig. 2.1B).

2.3.2 Zyflamend®-mediated induction of cell cycle inhibitors p21 and p27, mRNA and protein

In CWR22Rv1 cells, Zyflamend® treatment (24 hr) induced mRNA levels for the cell cycle inhibitors p21 and p27 (Fig. 2.2A). Concomitantly, protein levels of p21 and p27 were increased by 4-fold and 2-fold, respectively, with Zyflamend® treatment compared to controls (Fig. 2.2B). While p21 protein expression increased in the presence of Zyflamend®, p53 expression appears to be initially down regulated but these effects did not influence p21 (Fig. 2.2C). In addition, cyclin E was down-regulated with no change in cyclin D1 expression (data not shown). Changes in p21 protein levels were determined and relative changes in expression, turnover, or both were investigated. In the presence of Zyflamend® (24 hr), p21 protein expression was increased (lane 1 versus lane 2) (Fig. 2.2D). When Zyflamend® was removed (after 24 hr exposure), p21 levels did not change (lane 2 versus lane 4); however, when Zyflamend® was maintained for an additional 24 hr, protein level of p21 continued to increase (lane 2 versus lane 3), indicating Zyflamend® continuously induced p21 protein synthesis and/or prevented p21 protein degradation. To investigate p21 protein protection from degradation by Zyflamend®, cycloheximide (an inhibitor of protein synthesis) was added to the cells after 24-hr treatment with Zyflamend®; however, changes in p21 protein levels were not different in the control versus Zyflamend® treated cells (Fig. 2.2E).

A



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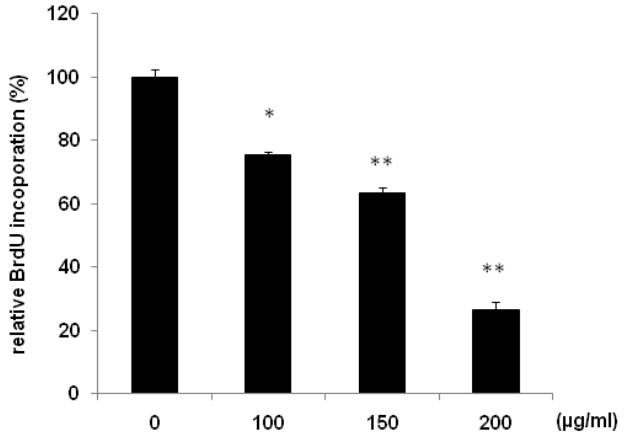

Figure 2.1 The effects of Zyflamend® on cell proliferation were determined using MTT or BrdU incorporation assays. A, RWPE-1, LNCaP, PC3 and CWR22Rv1 cells were treated ±Zyflamend® (0, 100, 150 and 200 ug/ml) over 24, 48, 72 and 96 hr. B, CWR22Rv1 cells were treated with Zyflamend® for 48 hr prior to the BrdU incorporation assay. Data are presented as mean ± SEM. *$p<0.05$, ** $p<0.005$.

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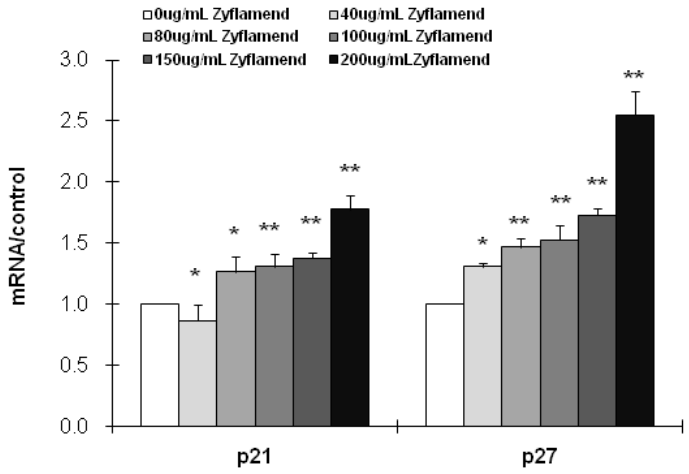

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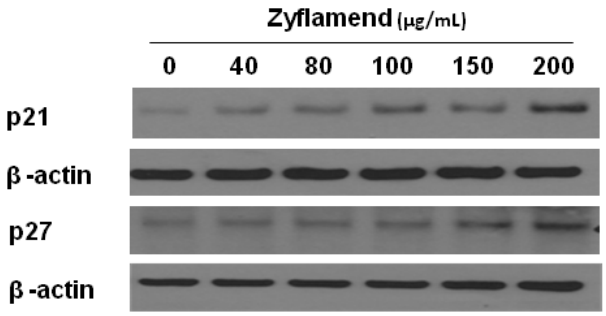
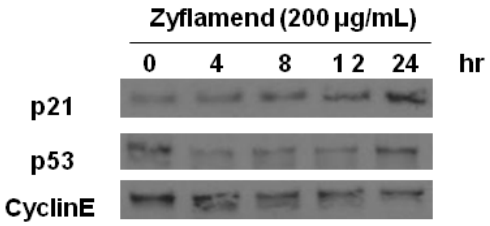


Figure 2.2 Effects of Zyflamend® on p21 expression using CWR22Rv1 cells. A, p21 and p27 mRNA levels (*$p<0.05$, ** $p<0.005$) were detected using qRT-PCR after 24 hr Zyflamend® treatment. B, p21 and p27 protein levels (24 hr treatment of Zyflamend®) using immunoblotting. C, protein levels of p21, p53, and cyclin E. D, p21 levels were detected in cells treated ±Zyflamend® after 24 hrs (lane 1: control; lane 2: +Zyflamend®); after 24 hr Zyflamend® treatment, cells were treated ±Zyflamend® for another 24 hr (lane 3: +Zyflamend®; lane 4: -Zyflamend®). E, CWR22Rv1 cells were treated with Zyflamend® for 24 hr prior to adding cycloheximide. Cells were then treated ±Zyflamend® for another 0-4 hr.

C



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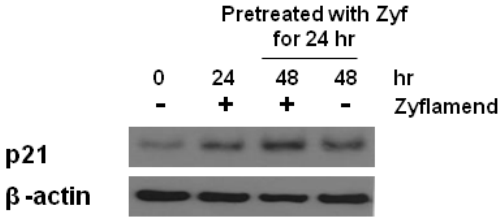

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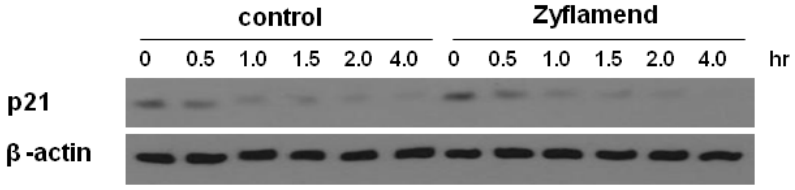

Figure 2.2 Continued.

2.3.3 p21 silencing induces cell growth

To link the molecular effects with biological outcomes, changes in p21 expression with cell growth were determined. CWR22Rv1 cells were transfected with siRNA against p21 in the presence or absence of Zyflamend®. Zyflamend® increased p21 mRNA expression in mock ($p<0.005$) and in negative control siRNA transfections ($p<0.05$) (Fig. 2.3A) with concomitant reductions in cell number (Fig. 2.3B). Transfection of p21 siRNA reduced p21 mRNA in the absence or presence of Zyflamend® (Fig. 2.3A). Comparing the mock/negative control groups to the p21 siRNA group in the presence of Zyflamend® (closed bars), there was a reduction in p21 mRNA levels with p21 siRNA treatment (Fig. 2.3A) and a concomitant increase in cell number (Fig. 2.3B). However, in cells not treated with Zyflamend® (open bars), cell numbers did not change following p21 siRNA treatment (Fig. 2.3B) despite reduced p21 expression below the baseline.

2.3.4 p21 overexpression reduced cell growth

To mimic the effect of Zyflamend®-induced p21, p21was overexpressed in CWR22Rv1 cells and confirmed by western blot (Fig. 2.3C). p21 protein levels were increased in cells with the p21 overexpression construct (lane 3) and in the presence of Zyflamend® (lane 2). The expression of p21 was further enhanced in the p21 stably transfected cells with the additon of Zyflamed® (lane 4). Both p21 overexpression and the presence of Zyflamend® reduced cell proliferation over time ($p<0.0005$). The reduction of cell proliferation by p21 overexpression was potentiated in the presence of Zyflamend® ($p<0.00005$, Fig 2.3D). These results were supported, in part, by the fact that Zyflamend® increases p21 promoter activity (data not shown) using a full length human p21 promoter (2.4kb) luciferase reporter construct, consistent with increases in mRNA and protein levels.

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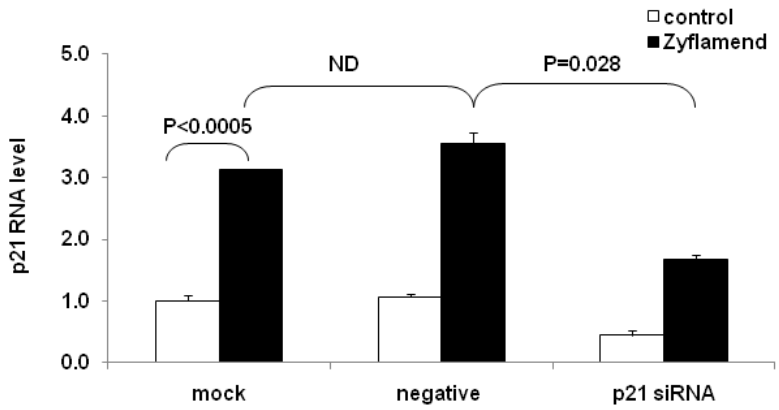

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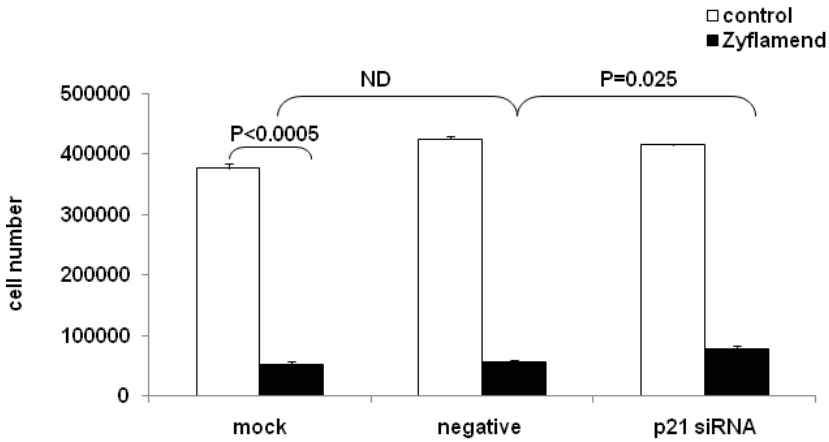
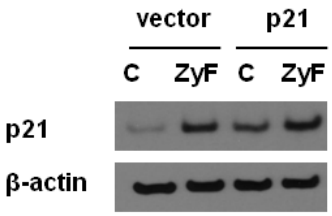


Figure 2.3 Effects of p21 on cell growth ±Zyflamend® using p21 silencing and p21 overexpression. A, p21 mRNA levels, and B, cell proliferation, were determined in the presence or absence of Zyflamend® (24 or 96 hrs, respectively) in cells transfected with mock (reagent only), negative (scramble SiRNA) and p21 SiRNA. Data are presented as the mean ± SD. C, p21 protein levels in cells transfected with vector or pRc/CMV-p21 treated ±Zyflamend® for 24 hr. D, Effects of Zyflamend® on cell proliferation in p21 or vector stably transfected cells using MTT assay. Data are presented as the mean ± SEM. **p<0.05, **p<0.005, †p<0.0005, ‡p<0.00005 and §p<0.000005*.

C



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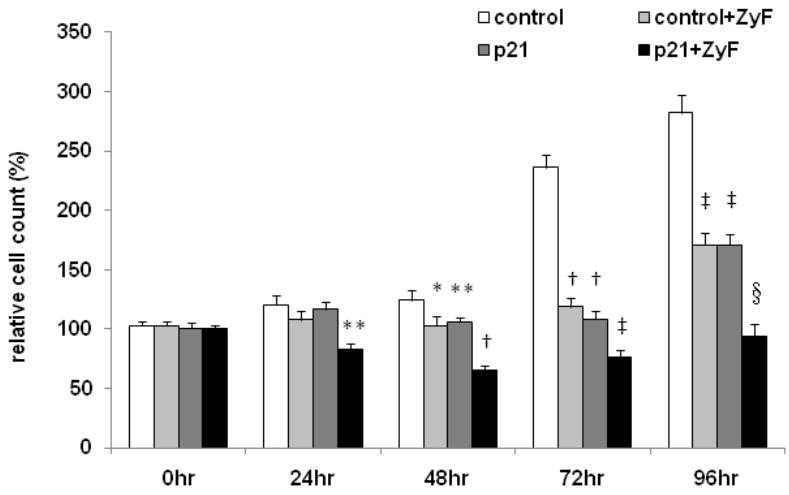


Figure 2.3 Continued.

2.3.5 p21 overexpression associated with cell growth reduction was significantly enhanced by Zyflamend®, but not individual herbs

Of interest was whether any of the individual herbal extracts had the same effect as the combined herbal extracts (i.e., Zyflamend®) (Fig. 2.4). We evaluated the effects of each herbal extract on p21 mRNA expression and cell proliferation using the same concentrations found in Zyflamend®. Individual herbal extracts or Zyflamend® were added to CWR22Rv1 cells for 24 and 48 hrs. Extracts of five herbs (barberry, goldthread, skullcap, green tea and ginger) significantly reduced p21 mRNA levels and three herbs (turmeric, hu zang and holy basil) significantly increased p21 mRNA levels; the extract from rosemery had no effect on p21 mRNA expression. Nevertheless, Zyflamend® had the most robust effect on increasing p21 mRNA levels ($p=0.0004$) (Fig. 2.4A) and was the only treatment that reduce cell proliferation (Fig. 2.4B).

2.3.6 Zyflamend®-mediates inhibition of HDACs

Because regulation of p21 expression has been linked to HDAC activity, the effects of Zyflamend® on class I and class II HDACs were investigated using CWR22Rv1 cells. In the presence of Zyflamend®, mRNA expression of all HDACs tested (HDAC1-7) was reduced by 30-80% (Fig. 2.5A). The reduction of HDAC protein levels was confirmed by western blot, and treatment with TSA (general HDAC inhibitor) was used as a control (Fig. 2.5B). Zyflamend® reduced protein levels (i.e., lane 1 versus lane 5) for HDAC1, HDAC2, HDAC3, and HDAC4, but not with HDAC5 or HDAC7. Treatment of CWR22Rv1 cells with TSA was shown to inhibit HDAC3 and HDAC4 expression in a dose-dependent manner (Fig. 2.5B). Concomitantly, Zyflamend® inhibited overall HDAC activity (nuclear extracts) (Fig. 2.5C). Since HDACs

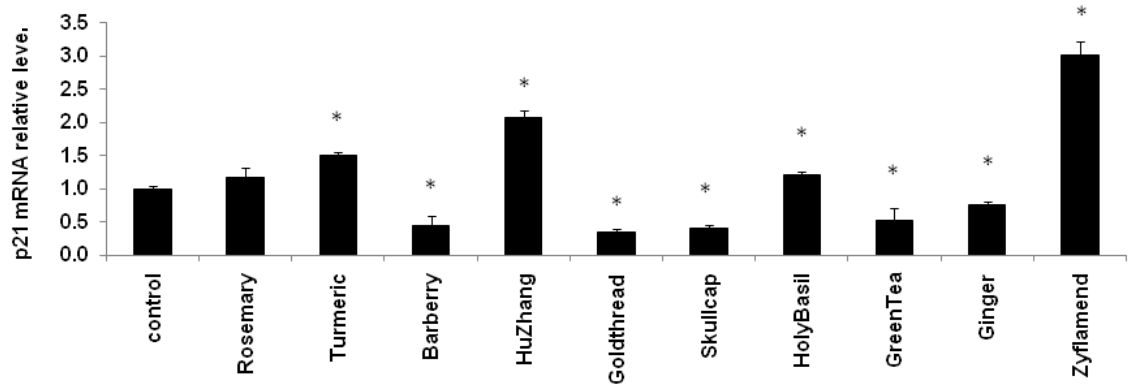
affect the structure of chromatin by removing acetyl groups from histones, thereby reducing transcriptional expression, histone acetylation was examined. Histone 3 acetylation was increased in the presence of TSA, Zyflamend® and the combination of TSA plus Zyflamend® (lane 1, lane 5 and lane 8) (Fig. 2.5D). Induction of p21 protein expression accompanied these changes, suggesting coordinated modulation of both events.

In addition, the effects of individual herbal extracts on HDAC expression were explored (Fig. 2.5E). Only Chinese goldthread and baikal skullcap appeared to mimic the down-regulation observed with Zyflamend® with regards to all HDACs tested. Extracts of rosemary, hu zang, holy basil, turmeric, green tea, barberry and ginger were unable to duplicate the effects observed with Zyflamend®.

2.3.7 Zyflamend®-induced p21 expression and induction of acetyl CBP/p300 via the MAPK pathway

To determine the involvement of mitogen-activated protein **(**MAP) kinases on p21 protein expression, we used the Erk inhibitor U0126 ± Zyflamend®. U0126 reduced Zyflamend®-induced p21expression (Fig. 2.6A). One target of particular interest was CBP/p300, transcriptional coactivators with histone acetyl transferase activity, and the down-stream target proteins of Erk and Elk-1. Zyflamend® increased the levels of phosphorylated Erk and Elk-1, and acetylated CBP/p300 (Fig. 2.6B) in a time-dependent manner.

A



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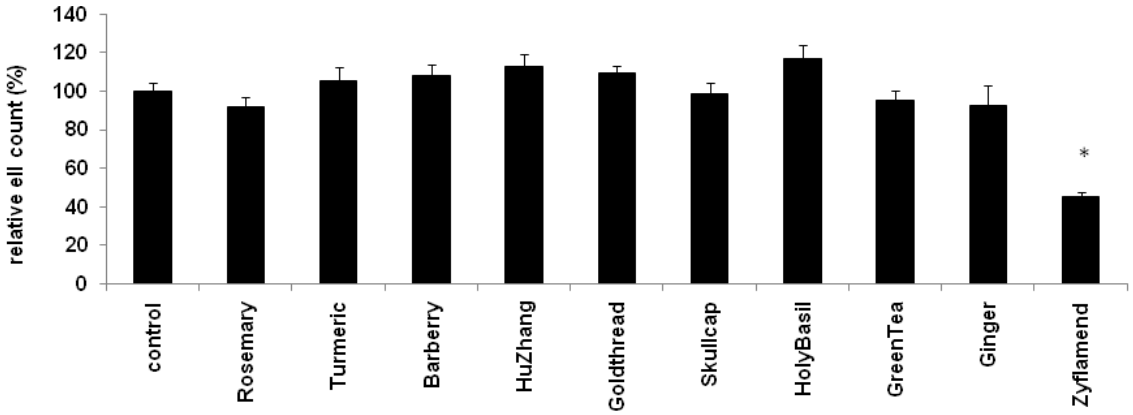


Figure 2.4 Effects of individual herbal extracts versus Zyflamend® on p21 mRNA expression and cell proliferation using CWR22Rv1 cells. A, Cells were treated with Zyflamend® or individual herbs at doses equivalent to those in Zyflamend® for 24 hr, and p21 mRNA was quantified by RT-PCR and normalized to control (* $p<0.05$). B, Cells were treated with Zyflamend® or individual herbal extracts for 48 hr and cell proliferation was measured by MTT assay (* $p<0.05$).

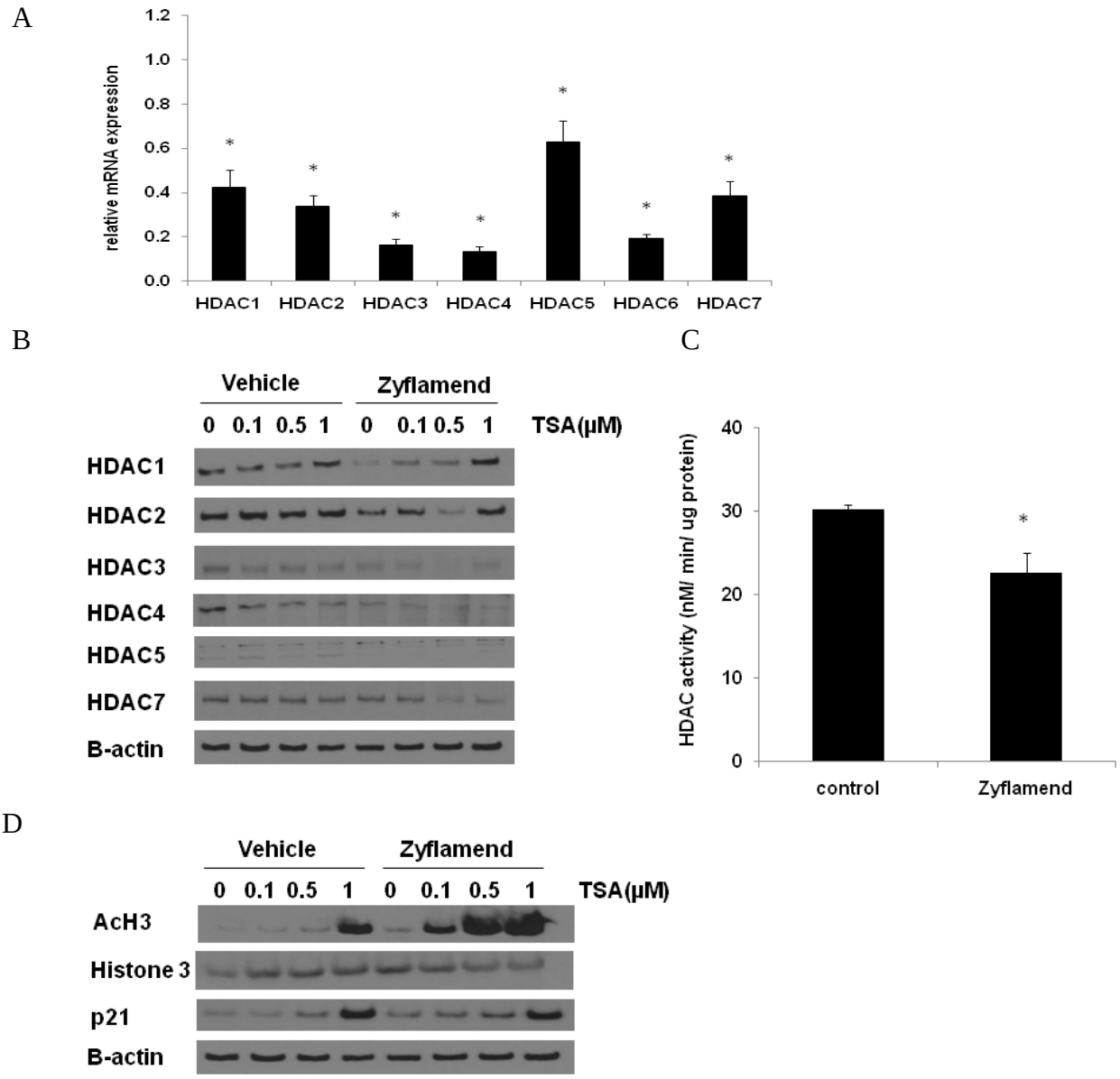
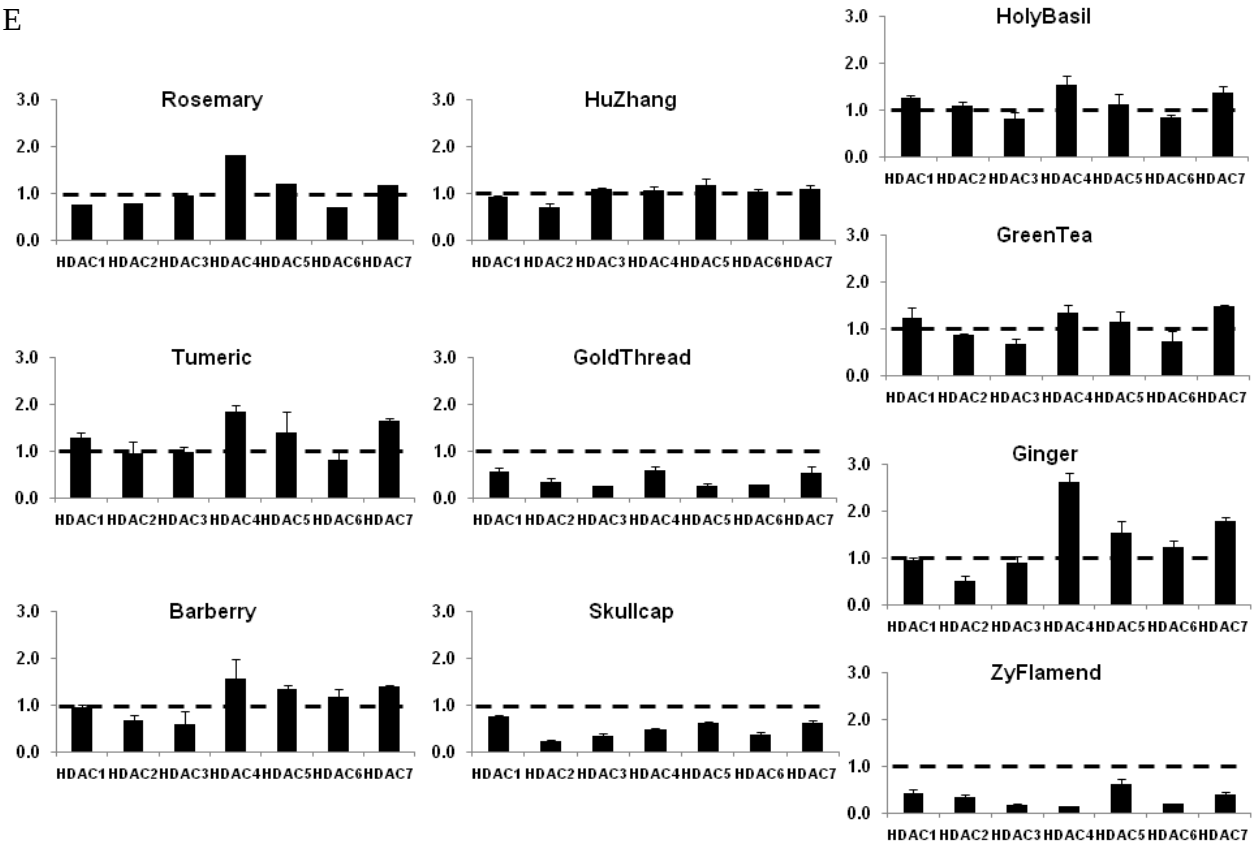


Figure 2.5 Class I and class II histone deacetylases mRNA, protein and activity were determined ±Zyflamend® in CWR22Rv1 cells. A, HDACs mRNA levels after treatment with Zyflamend® for 24 hr. B, HDAC proteins levels ±Zyflamend®, ±TSA after 24 hr treatment. C, Effects of Zyflamend® on nuclear HDAC activity (nM/min/ug protein). D, Acetyl histone 3 (AcH3), histone 3 and p21 protein levels ±Zyflamend®, ±TSA after 24 hr. E. Effects of individual herbal extracts on mRNA levels of various histone deacetylases.

E



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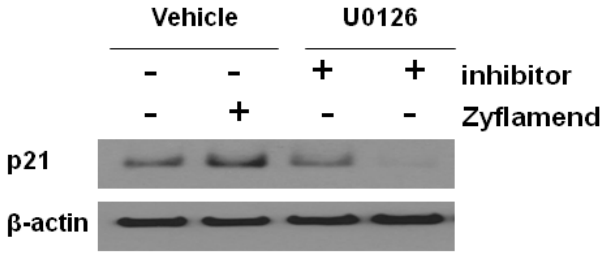

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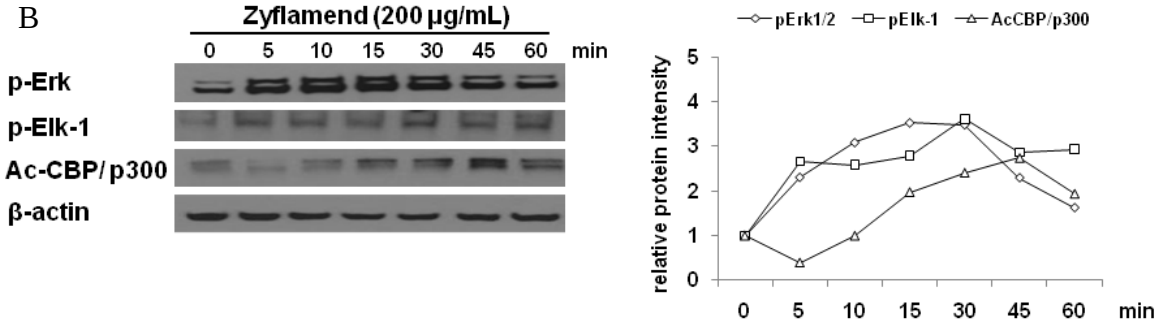

Figure 2.6 Effects of Zyflamend® on the mitogen-activated protein kinase (MAPK) pathway and p21 expression. A, Effect of Zyflamend® on p21 protein expression in the presence or absence of the Erk inbibitor U0126 (2 uM). B, Effects of Zyflamend® (0-60 min treatment) on protein expression of p-Erk, p-Elk-1 and acetyl-CBP/p300. C. A model of epigenetic regulation by Zyflamend® on p21 expression by activation of histone acetyl transferase (CBP/p300) through MAPK pathway and inhibition of histone deacetylases reducing cell proliferation.

C

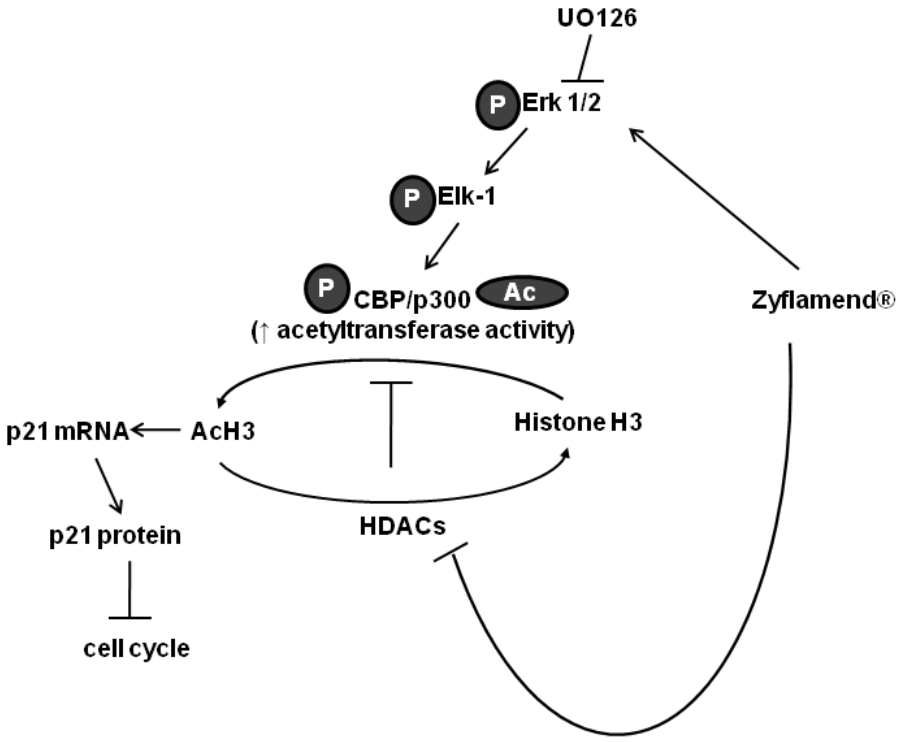

Figure 2.6 Continued.

2.4 Discussion

The use of herbs and botanicals and their bioactive components are effective inhibiting growth, angiogenesis, metastasis and inducing apoptosis in many tumor lines. Many of their molecular mechanisms of action have been characterized *in vitro* (12, 5, 13). In addition, the use of herbal extracts in combination appears to potentiate each other's actions, resulting in significant activity whereas single agents may have greatly reduced effects (7, 14).

Zyflamend® is derived from the extracts of ten different herbs, each with reported medicinal properties. Individuals diagnosed with HGPIN are at increased risk for developing PrC (15). When this polyherbal preparation was used in a Phase I clinical trial with patients diagnosed with HGPIN, reductions in PSA and re-diagnosis to benign forms of prostate tissue were reported (8, 16). Based on this data, we investigated the effects of Zyflamend® in a cell model of castrate-resistant PrC with a particular interest in identifying molecular mechanisms of action.

To understand the doses used in this study, we chose a maximum dose of Zyflamend® based on the levels of curcumoids (~7% w/w in turmeric (17)) found in the poly-mixture. This level of curcumoids would be equivalent to the identifiable range of human plasma concentrations for Zyflamend® at these physiologically relevant concentrations inhibited PrC cell growth in concentration- and time-dependent manners. These effects were observed in RWPE-1 cells (immortalized prostate epithelial cells that represent an early stage of the disease, i.e., PIN (18)), and in tumor cell lines representing androgen-dependent (LNCaP), androgen-independent (PC3), and castrate-resistant (CWR22Rv1) forms of the disease. Similar results with Zyflamend® were observed by others using PC3 and LNCaP cell lines (9, 11). Because of our

previous interest of investigating castrate-resistant PrC (19), we focused our attention on those underlying molecular mechanisms responsible for these effects using CWR22Rv1 cells.

Zyflamend® increased cell cycle regulators p21 and p27, duplicating previous results of Zyflamend® on p21 expression (9, 11). P21 and p27 are common targets for bioactive phytonutrients (7, 20). Zyflamend® consistently enhanced mRNA and protein levels in dose- and time-dependent manners. These effects were always biologically correlated with reductions in cell proliferation. Increases in p21 protein levels were due to increases in transcription and not a reduction in protein stability. Transcriptional expression of p21 can be regulated by p53-dependent (21-23) and/or p53-independent (24-31) mechanisms. We observed that Zyflamend®-mediated increases in p21 were not accompanied by concomitant changes in p53 expression. We also observed reductions in the expression of cyclin E, a protein inhibited by p21 and involved in regulating the transition of the G1/S phase of the cell cycle. In addition to these effects, Zyflamend® has been reported to modify other cell cycle regulators, including retinoblastoma protein (phosphorylation) and cyclin D1 in PC3 cells (11).

To confirm that Zyflamend® was attenuating cell proliferation, in part, by up-regulating p21 expression, we used siRNA technology to silence p21 and stably transfected cells for over expression. Overexpression of p21 significantly inhibited proliferation, duplicating those effects observed with Zyflamend®. Knocking down p21 mRNA levels in the absence of Zyflamend® had no effect on cell proliferation, suggesting the basal level of p21 was not acting in a regulatory manner. However, knocking down Zyflamend®-induced p21 mRNA levels increased cell proliferation, reaffirming that the effects of Zyflamend® are, in part, mediated by p21 upregulation. Importantly, when Zyflamend® was added to cells overexpressing p21, there was

an added reduction in cell proliferation, suggesting multiple mechanisms may be involved. This makes sense since Zyflamend® is comprised of 10 different herbal extracts.

Induction of p21 expression by the individual herbal extracts was quite variable. However, cell proliferation was unaffected by any of the herbal extracts when used individually at concentrations present in the Zyflamend® preparation; only the combination proved to be effective. Levels of each extract when used individually could have been below the physiologically active threshold, accounting for the lack of effects on cell proliferation (as discussed in Ref (5)).

Post-translational modification of histones, in particular the removal or addition of acetyl groups on ε-N-acetyl lysine residues, play an important role in epigenetic regulation of transcription (32). Acetylation of the N-terminal tails of histones "relaxes" the chromatin making it more accessible for binding by coactivating factors. The result is an increase in gene expression. In contrast, deacetylation results in a more compact chromatin and transcriptional repression. Regulation of acetylation is a balance between histone acetyl transferases and histone deacetylases (HDACs) (33). HDACs in particular are important in cancer biology, by promoting proliferation, angiogenesis, migration/metastasis, resistance to chemotherapy, and inhibiting apoptosis and differentiation (as reviewed in Ref (34)). Identification of HDAC inhibitors is therefore a new therapeutic approach to treat cancer (35).

Epigenetic regulation via acetylation is important in regulating tumor suppressor genes, such as p21 (36). Zyflamend® increased acetylation of histone 3, in part, by down regulating mRNA levels of class I and class II HDACs (HDAC1-7), reducing protein expression of HDAC1, HDAC2 and HDAC4, and inhibiting HDAC activity. HDAC1 and HDAC4 have been shown to

be particularly specific for the repression of p21 expression (37, 36, 38). Hyper-acetylation of Sp1 binding sites in the proximal promoter is a key regulator of p21 expression (36) and HDAC4 has been shown to regulate expression through a Sp1-dependent, p53-independent pathway (37).

In contrast to Zyflamend®, when cells were treated with the individual herbs, only goldthread and skullcap reduced HDAC expression, but interestingly p21 expression was down regulated in the presence of either of these herbal extracts. There is increasing evidence that many bioactive phytochemicals may function as histone deacetylases inhibitors, but these results typically are generated with concentrations many times higher than what was used here (39).

Up regulation of p21 expression also is coordinately regulated by CBP/p300. CBP/p300 proteins are transcriptional coactivators with histone acetyltransferase activity that is involved in the regulation of p21 expression through the Sp1 binding sites (40-41). The histone acetyltransferase activity of CBP/p300 is increased by phosphorylation and is regulated upstream by Erk-1/-2 and its downstream regulator, Elk-1 (42). MAP kinase-dependent phosphorylation of Elk-1 results in interaction with p300 and increased histone acetyltransferase activity (43). In a time-dependent manner, Zyflamend® increased the expression of pErk and pElk-1, followed by the acetylated form of CBP/p300 (44). MAP kinase involvement was confirmed using the Erk-1/-2 inhibitor U0126 which resulted in the down regulation of pErk in a time dependent manner (data not shown) and inhibition of Zyflamend®-induced p21 expression. Stimulation of p21 expression via up regulation of the Erk pathway has been observed by others and these effects were similarly blocked in the presence of the Erk-1/-2 inhibitor U0126 (45).

In summary, Zyflamend® is composed of ten concentrated herbal extracts. It has been used successfully in a Phase I clinical trial (8), suggesting it may be helpful for patients diagnosed with PIN, which is related to development of PrC. These studies suggest it also may have utility in addressing advanced forms of PrC. The concentrations used in this study were of importance and carefully considered. When compared to the multi-herbal preparation, the use of the herbal extracts in isolation could not duplicate the overall effects of Zyflamend®, and this may be related to those concentrations used. Zyflamend inhibited tumor cell growth *in vitro*, in part, by up-regulating the tumor suppressor protein p21. The epigenetic regulation of p21 by Zyflamend® can be summarized in Figure 2.6 C. Increased expression of p21 was coordinately regulated by activation of the histone acetyltransferase CBP/p300 through up regulation of pErk signaling and through concomitant down regulation of class I and class II HDACs. These effects modified histone hyper-acetylation, a key activator of p21 expression and cell cycle regulation.

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Chapter III

Zyflamend® Reduces the Expression of Androgen Receptor and Insulin-Like Growth Factor Receptor, Biomarkers of Cancer Relapse, in a Model of Castrate-Resistant Prostate Cancer

Abstract

Prostate cancer is the most commonly diagnosed solid malignancy and tumor cells eventually transform to castrate-resistant or androgen-independent relapse through the proposed activation of the androgen receptor via insulin-like growth factor receptor (IGF-1R) signaling involving phospho-AKT (pAKT). In this study, a mixture of medicinal herbal extracts, Zyflamend®, was used as an adjuvant treatment in a model of castrate-resistant prostate cancer, CWR22Rv1 cells. We found that Zyflamend® reduced androgen receptor and IGF-1R expression along with a reduction of IGF-1-mediated proliferation of CWR22Rv1 cells. To investigate the involvement of pAKT in the downstream signaling of IGF-1, IGF-1 activated AKT phosphorylation; however, the induction of pAKT was not associated with androgen receptor expression. Further, constitutive active form of AKT had no effect on nuclear expression of androgen receptor, indicating that upregulation of pAKT did not promote androgen receptor expression or nuclear translocation in castrate-resistant CWR22Rv1 cells. Conversely, Zyflamend® reduced androgen receptor expression following IGF-1 stimulation and in cells over-expressing pAKT. These results demonstrated that Zyflamend® inhibited IGF-1-stimulated cell growth, IGF-1R expression and androgen receptor expression and its nuclear localization, but these effects were not mediated through PI3K/pAKT signaling. In addition to an inhibition of androgen receptor and its growth promoting effects by Zyflamend®, it was found that Zyflamend® influenced indices of proliferation and apoptosis by inducing caspase 3 activity, PARP cleavage, and inhibiting Bcl-2 expression and the proliferative biomarker Ki67. In conclusion, Zyflamend® inhibited IGF-1R and androgen receptor expression in a PI3K/pAKT independent manner. Furthermore, some of Zyflamend's effects involve a concomitant decrease in cell proliferation and an increase in programmed cell death.

3.1. Introduction

Prostate cancer is the most commonly diagnosed solid malignancy. In the last few years, prostate cancer has become the second leading cause of cancer-related deaths in men in the United States (1-2). The advanced forms of prostate cancer metastasize to distant organs, most commonly bone, and eventually become castrate-resistant or androgen-independent following androgen deprivation (commonly referred to as androgen deprivation or hormone ablation therapy) (3). This advanced stage of the disease is very difficult to treat and is invariably fatal.

Zyflamend® is a poly herbal preparation consisting of the supercritical CO_2 fluid extracts of ten different medicinal herbs shown to have anti-neoplastic activity. In phase I clinical trials, Zyflamend® has demonstrated efficacy in patients with high-grade prostatic intraepithelial neoplasia (HG-PIN), a premalignant form of the disease (4), and has inhibited the growth of androgen-dependent, androgen-independent and castrate-resistant prostate cancer cells *in vitro* and *in vivo* via multiple mechanisms (5-6). These latter results suggest that Zyflamend® could potentially have efficacy in metastatic forms of this disease, a possible adjuvant to hormone deprivation therapy**.** The mechanisms of prostate cancer cell resistance to androgen withdrawal have been investigated and one mechanism that is repeatedly mentioned as a potential target is reactivation of androgen signaling in the absence of androgens (7). Amplification of androgen receptor expression (8) and activation of androgen signaling, in the absence or presence of very low levels of androgens, may involve crosstalk with other signaling pathways (9-10). For example, androgen receptor signaling can be activated via crosstalk between, insulin-like growth factor-1 (IGF-1)/ insulin-like growth factor-1 receptor (IGF-1R) signaling. Levels of IGF-1 are significantly higher in metastatic prostate cancer patients when compared with the controls (11) and IGF-1/IGF-1R signaling have been shown to

activate the androgen receptor in the presence of low levels of androgens (12).Similarly, the expression of IGF-1R is increased in patients with progressive forms of prostatic neoplasia (13), demonstrating a correlation of IGF-1/IGF-1R and prostate cancer progression. The mechanisms of IGF-1/IGF-1R on androgen receptor activation have linked IGF-1/IGF-1R signaling to increased androgen receptor activity through phosphatidylinositol 3-kinase (PI3K)/AKT phosphorylation (12, 14). The PI3K/AKT pathway is reported to activate androgen receptor signaling via the dissociation of a suppressor-androgen receptor complex, thus allowing androgen receptor to bind to the promoter regions of target genes such as prostate specific antigen (PSA) (15-16) and cell proliferative promoting growth factors such as IGF-1 (17), forming a positive feedback of androgen receptor and IGF-1 signaling. However, this pathway has yet to be studied in a model of castrate-resistant prostate cancer using cells that are more in-line with the human disease, those that express an active androgen receptor, secrete human PSA and can grow in an androgen-deprived environment.

In this study, we used a combination of herbal extracts, Zyflamend®, as an adjuvant therapy in an experimental model of castrate-resistance using a the human prostate cancer cell line, CWR22Rv1. Zyflamend® has been found to inhibit androgen receptor expression in an androgen-dependent prostate cancer cell line, LNCaP (5), but the mechanisms were not investigated. In this study, we found that Zyflamend® reduced protein levels of IGF-1R and androgen receptor in CWR22Rv1 cells and investigated whether crosstalk between these pathways involved PI3K/AKT signaling.

3.2. Materials and Methods

3.2.1 Cell Culture

Human prostate cancer cell line, CWR22Rv1, was purchased from American Type Culture Collection (Rockville, MD). CWR22Rv1 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum under an atmosphere of 5% CO_2 at 37^{o}C. Cells were harvested with the addition of 0.25% trypsin with 0.02% EDTA during the exponential growth phase. For the experimental treatments, cells were cultured in RPMI 1640 media supplemented with 0.05% fetal bovine serum containing Zyflamend® (New Chapter, Inc, Brattleboro, VT) reconstituted in dimethyl sulfoxide (DMSO) for cell proliferation assay, mRNA extraction and protein isolation. In all experiments, 0.1% DMSO was used as the vehicle control. CWR22Rv1 cells are hormone independent and represent a very late stage of prostate cancer following hormone ablation. On the other hand, CWR22Rv1 cells produce PSA and express a functional androgen receptor. These cells can grow in the presence or absence of androgens and are considered to be a castrate-resistant cell line (18). CWR22Rv1 cells were chosen for most of the experiments because prostate cancer cells in most patients who relapse following androgen ablation therapy express androgen receptor and produce PSA.

3.2.2 Cell Proliferation

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Andwin Scientific, Addison, IL) is cleaved by metabolically active cells to form a formazan, chromaphore product that absorbs at 540 nm. The MTT assay was used to assess relative cell growth and viability, following the manufacturer's instructions (Promega, Madison, WI). Cells (1×10^4 cells of CWR22Rv1) were plated in 96-well plates in a volume of 100 ul culture medium. The culture medium contained 200 ug/ml of Zyflamend® in the presence or absence of insulin

growth-like factor-1 (100 ng/ ml). 0.1% of DMSO was used as the controls. For PI3K inhibitor experiments, cells were treated wortmannin (1.0 μM). Cell proliferation was determined at 0, 24, 48, 72, 96 hr post incubation. At each time point, a mixture of MTT:complete medium (1:10, v/v) was added and incubated at 37 ^{o}C for 4 hr in a CO_2 incubator (5%). The absorbance was measured on a SpectraCount microplate photometer (Perkin Elmer Inc, Waltham, MA).

3.2.3 Induction of pAKT by Adenovirus infection

CWR22Rv1 cells (1.5×10^5 cells per well in 6-well plates) were plated overnight prior to adenovirus infection. The myrAkt1 adenovirus N-terminally myristoylation signal-attached Akt (myr-Akt) expresses constitutive active AKT1 protein described previously (19). CWR22Rv1 cells in RPMI 1640 media containing 10% FBS were infected with the myrAKT1 adenovirus for 6 hr, after which time the cells were cultured overnight in RPMI 1640 media containing 0.5% FBS while β-Gal constructed adenovirus was used as the control. Infected cells were pre-treated with vehicle or Zyflamend® (200 ug/ml) for 24 hr at 37 ^{o}C. Protein expression of pAKT (serine 473), AKT and androgen receptor was examined by western blot.

3.2.4 Western blotting

CWR22Rv1 cells were lysed in the presence of cell lysis buffer (Cell Signaling Technology®, Inc. Danver, MA). Protein concentrations of the lysates were quantified by BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Lysates (15 ug) were fractioned by 8~12% SDS-PAGE and transferred to a polyvinylidine diflouride (PVDF) membrane by electroblotting. The membranes were blocked using 5% nonfat dry milk in 0.1% Tris buffered saline-Tween-20 (TBST) for 1 hour and were incubated with primary antibodies overnight at 4°C. The membrane was incubated with anti-mouse or anti-rabbit secondary antibody conjugated with

horseadish peroxidase (HRP) (Cell Signaling Technology®, Inc. Danver, MA). Protein expression was detected with a Pierce ECL Western Blotting detection system. Each membrane was exposed to Hyperfilm Film (GE Healthcare, Piscataway, NJ). Antibodies of AR, phospho-ser473-AKT, AKT, cleaved caspase 3, poly (ADP-ribose) polymerase, p65 (Cell Signaling Technology®, Inc. Danver, MA), IGF-1Rα and Ki67 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used. β-actin with anti-β-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as the control.

3.2.5 Immunofluorescence staining

CWR22Rv1 cells were seeded on the cover slips supplemented in RPMI 1640 media with 10% FBS under an atmosphere of 5% CO_2 at 37^{o}C overnight. Before the treatment, CWR22Rv1 cells were maintained in RPMI 1640 media with 0.5% FBS. For the observation of androgen receptor translocation, the cells were pretreated with Zyflamend® (200 ug/ml) for 24 hr. After the treatment, the cells were fixed using 2% paraformaldehyde for 15 min, followed by blocking with 10% goat serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1hr, and anti-androgen receptor antibody (1 μg) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 ^{o}C. Alexa Fluor® 488 goat anti-rabbit IgG was used as secondary antibody.

3.2.6 Caspase 3/7 activity assay

CWR22Rv1 cells were lysed in cell lysis buffer after treated with Zyflamend®. Fifty μg of cell lysates were used for the Caspase 3/7 Assay (Promega, Madison, WI). Briefly, 50 μg of cell lysates in 50 μl lysis buffer were mixed with 50 μl of caspase 3/7 reagent and incubated in dark for 1 hr at room temperature. Caspase 3/7 activity was measured on a FluoroCount microplate photometer (Perkin Elmer Inc, Waltham, MA) at a 485 nm excitation and a 527 nm

emission.

3.2.7 Protein degradation

CWR22Rv1 cells were cultured with RPMI 1640 medium containing Zyflamend® (200 μg/ml) for 24 hr prior to adding cycloheximide (10 μM) to terminate protein synthesis for an additional 0, 0.5, 1, 1.5, 2, 4 hr in the continued presence or absence of Zyflamend® (200 μg/ml) and harvested for protein analysis.

3.2.8 RNA isolation and quantitative RT-PCR

Total RNA was isolated from CWR22Rv1 cells using Trizol reagent (Invitrogen, Carlsbad, CA) followed by chloroform extraction. The aqueous phase was precipitated in 100% isopropanol and the pellet was washed in 75% ethanol prior to re-suspension in RNase-free water. Contaminating DNA was removed from RNA samples using Turbo DNA free™ kit (Ambion, Inc. Austin, TX) and then the concentration of total RNA was measured using NanoDrop 1000™ (Thermo Scientific Wilmington, DE). Total RNA (2 μg) from each sample was mixed with MultiScribe™ Reverse Transcriptase, RNase Inhibitor, dNTP Mixture, random hexamers, RT buffer, $MgCl_2$ solution and incubated at 25℃ for 10 min, 48℃ for 30 min and 95℃ for 5 min to reverse transcribe to cDNA using TaqMan® reagent kit (Applied Biosystems, Carlsbad, CA). cDNA samples were used for quantitative RT-PCR (ABI Prism® 7300 Real-Time PCR System, Applied Biosystems, Carlsbad, CA). cDNA (0.7μl) was used as a template for qPCR amplification with primer sets (2.5 μM) of androgen receptor sense, 5'-CCTGGCTTCCGCAACTTACAC-3' and antisense 5'- GGACTTGTGCATGCGGTACTCA-3', and prostatic specific antigen sense, 5' - ACCAGAGGAGTTCTTGACCCCAAA-3' and antisense 5'- CCCCAGAATCACCCGAGCAG-3' were examined. Amplification was performed

using a standard thermocycle program beginning with an initial temperature at 94°C for 1 min followed by 30 cycles of 94°C for 15 sec, 50°C for 30 sec and 72°C for 2 min. Each sample was examined in triplicate and the amounts of PCR product were normalized with 36B4, 5'-TGCATCAGTACCCCATTCTATCA-3' and 5'-AAGGTGTAATCCGTCTCCACAGA-3' as the internal control.

3.2.9 Statistical analysis

The results are presented as mean ± SD and multiple comparisons are analyzed by a two-way ANOVA. Differences were considered significant at $p<0.05$.

3.3. Result

3.3.1 Effect of Zyflamend® on the growth of castrate-resistant prostate cancer CWR22Rv1 cells

Zyflamend® inhibited growth of castrate-resistant prostate cancer cells, CWR22Rv1 cells, in a time-dependent manner. After 48 hr treatment, Zyflamend® significantly inhibited cell growth by 38% ($p<0.005$), and cell growth was further reduced by 70% ($p<0.005$) after a 96 hr treatment (Figure 3.1).

3.3.2 Zyflamend® and androgen receptor and prostate specific antigen expression

Androgen receptor in CWR22Rv1 cells was detected via immunofluorescent imaging following 24 hr treatment of Zyflamend®. The green fluorescent stain corresponds to protein intensity. Zyflamend® reduced androgen receptor intensity as measured via immunofluorescent staining (Figure 3.2A). This result was confirmed with western blot analysis (Figure 3.2A). Concomitantly, androgen receptor and prostate specific antigen mRNA levels were decreased by

greater than 65%. However, androgen receptor degradation was not promoted by Zyflamend® (Figure 3.2 C) when protein synthesis was blocked by cycloheximide.

3.3.3 Zyflamend® and its effect on cell proliferation in the presence or absence of IGF-1

CWR22Rv1 cells were treated with IGF-1 (10 and 100 ng/ml) with and without Zyflamend® (200 µg/ml) for up to 96 hrs. IGF-1 increased cell proliferation in a dose-dependent manner and these effects were significantly attenuated in the presence of Zyflamend® (Figure 3.3B). These results were consistent with a reduction in the protein levels of IGF-1R (Figure 3.3C). Cultured cell morphology was monitored over 96 hr following treatment with vehicle (control), IGF-1, Zyflamend® and a combination of IGF-1 and Zyflamend® (Figure 3.3D). IGF-1 increased cell density compared to the control cells after 24 hr treatment. Conversely, cells started to shrink, round-up, and detach with treatment of Zyflamend® at 24 hr compared to the control and IGF-1-treated cells (Figure 3.3E). This affect of Zyflamend® was delayed by IGF-1, but by 48 hr cells exposed to the combination of IGF-1 and Zyflamend® were similar to Zyflamend® alone. After 96 hr IGF-1 co-treatment had no observable affect in combination with Zyflamend®.

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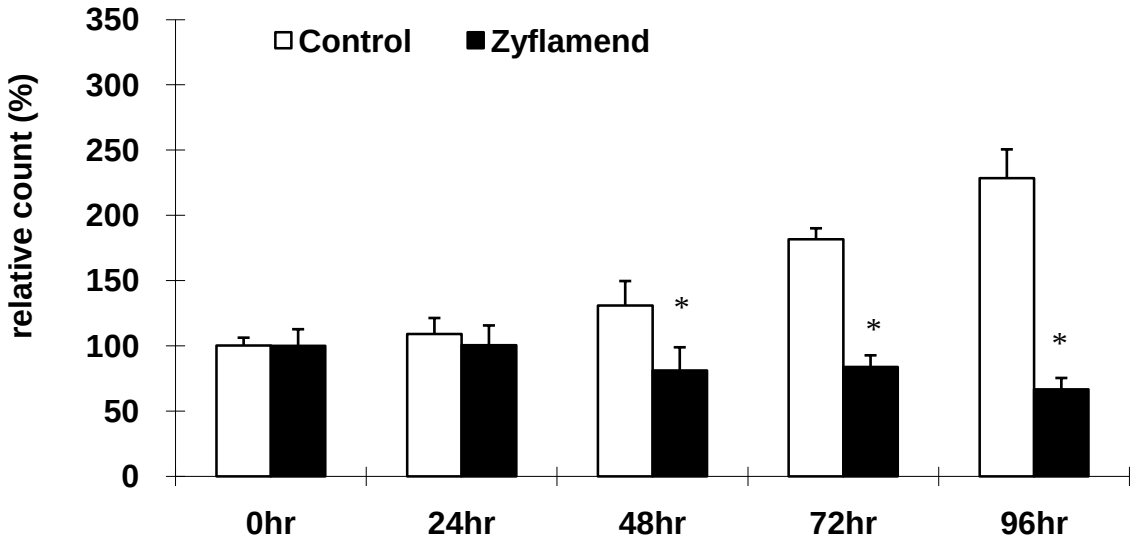

Figure 3.1 The effect of Zyflamend on cell proliferation. CWR22Rv1 cells were treated with Zyflamend® (200 μg/ ml) for 0-96 hr and cell proliferation was compared to control using MTT assay. Values are means±SEM (n=8/group). Differences were considered significant at $p<0.05$ as compared to control.

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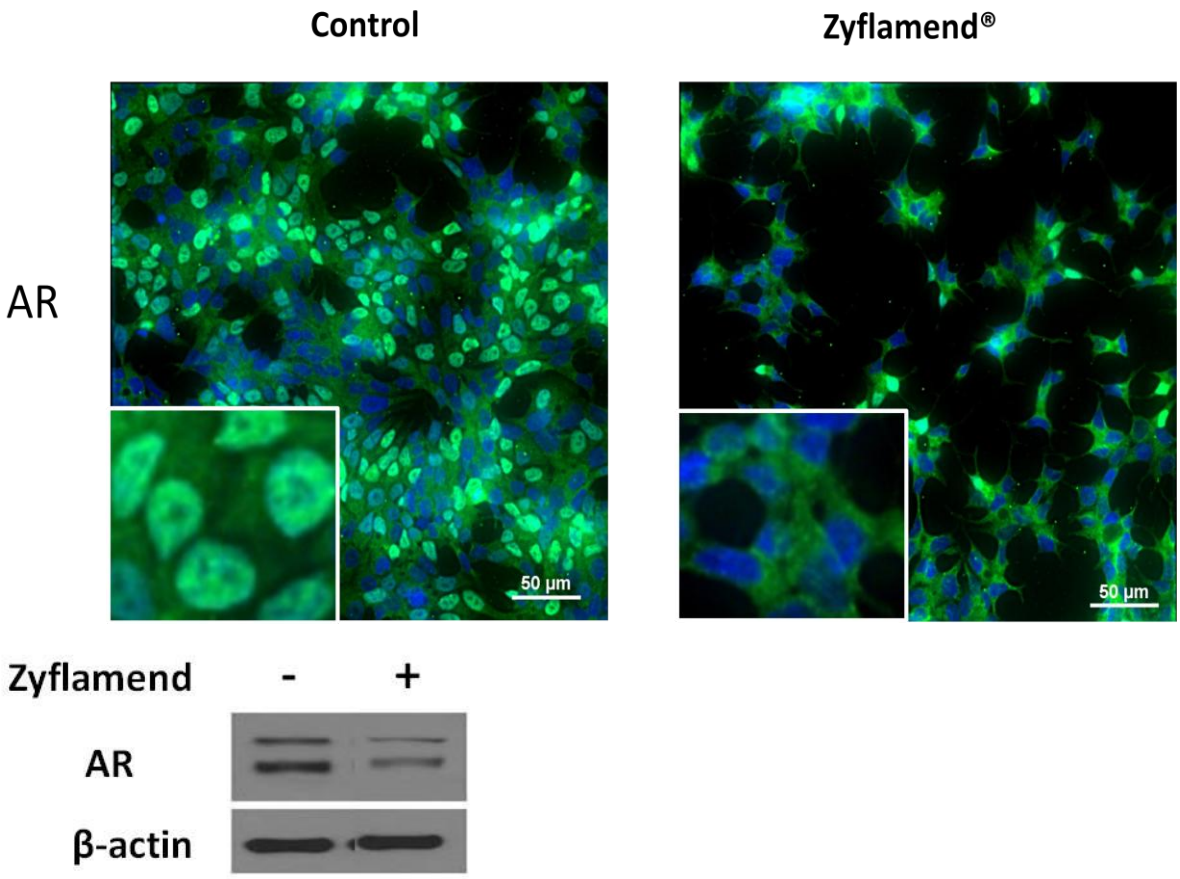
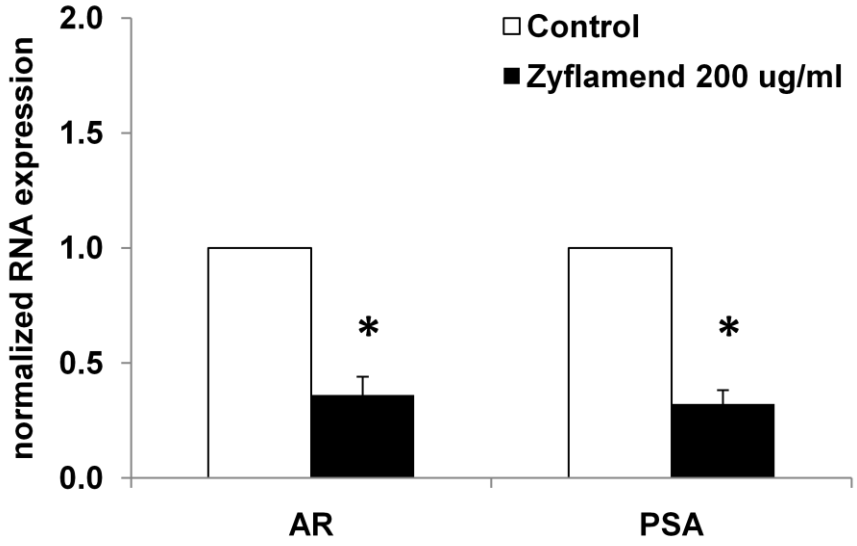


Figure 3.2 Effect of Zyflamend® on androgen receptor (AR) and prostate specific antigen (PSA) expression. A. Zyflamend® (200 µg/ml) was added to the cells for 24 hr and the expression of AR was detected using immunofluorescent staining and protein expression via western blot analysis. B. Expression of mRNA levels of AR and PSA were detected using RT-PCR following 24 hr-treatment of Zyflamend® (200 µg/ml). Values are means±SEM (n=3/group) and considered significant at $p<0.05$ as compared to the control. C. AR synthesis was blocked using cycloheximide for 4 hrs with or without Zyflamend® and levels of AR were monitored via western blot analysis.

B



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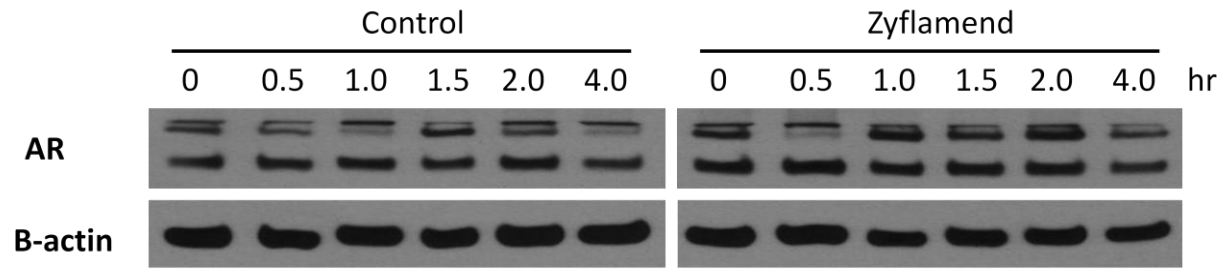

Figure 3.2 Continued.

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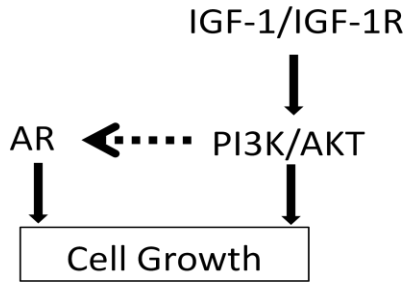

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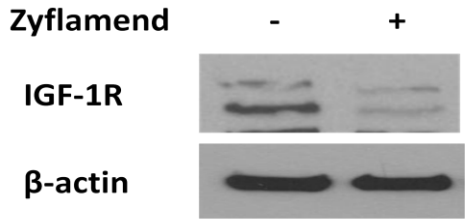

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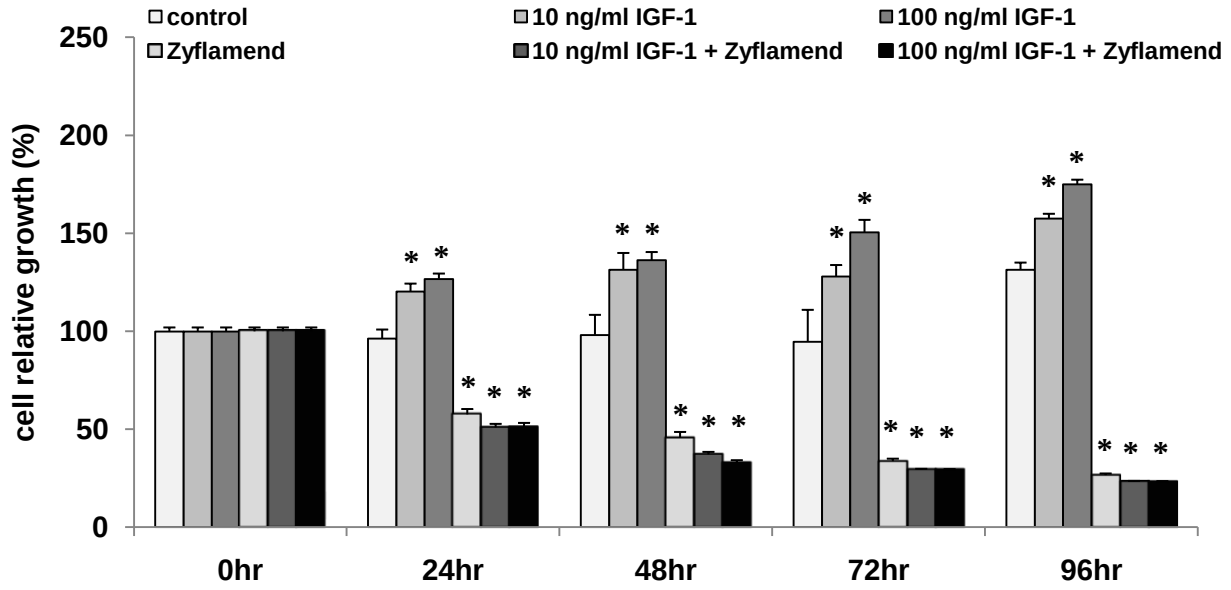
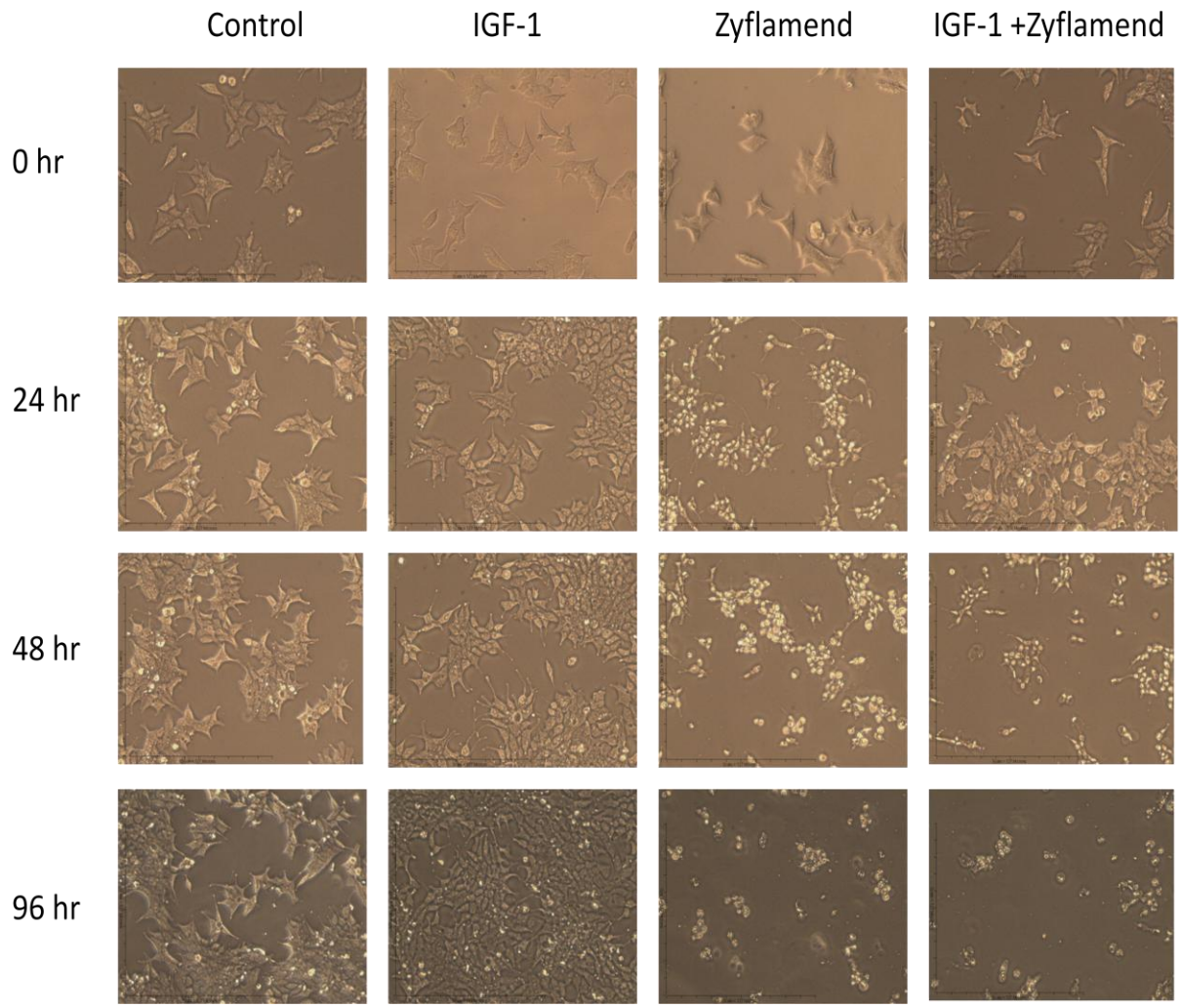


Figure 3.3 Effect of Zyflamend® on IGF-1R expression and cell proliferation in the presence or absence of IGF-1. A. This figure presents the signaling model where IGF-1/IGF-1R potentially promotes cell growth via pathways dependent upon or independent of androgen receptor. B. Cell proliferation (0-96 hr) in the presence or absence of IGF-1 (10 and 100 ng/ml) and Zyflamend (200 μg/ml). Values are means±SEM (n=8/group) and considered significant at $p<0.05$ as compared to control. C. IGF-1R expression levels (protein) following 24 hr treatment of Zyflamend® (200μg/ml). D. Effect of IGF-1 on morphological changes in CWR22Rv1 cells in the presence or absence of Zyflamend® after treatment for 96 hrs. E. Enlarged images of CWR22Rv1 cells following 24hr treatment of vehicle or Zyflamend® in the presence or absence of IGF-1.

D



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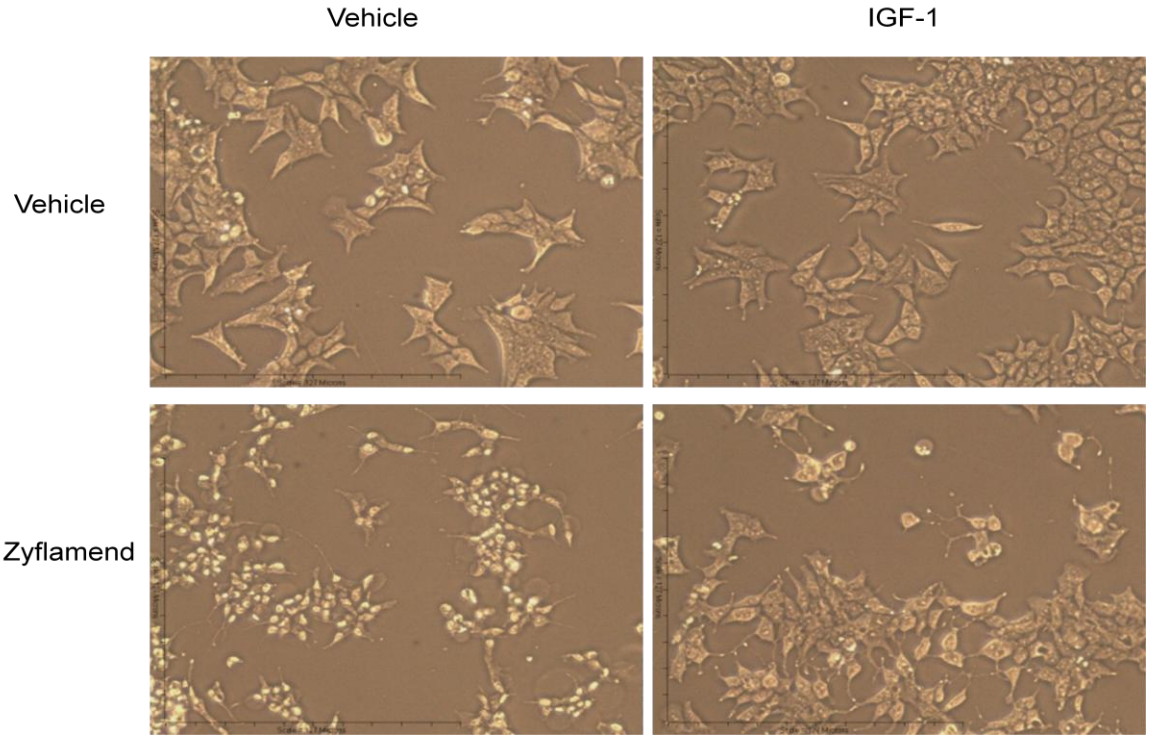


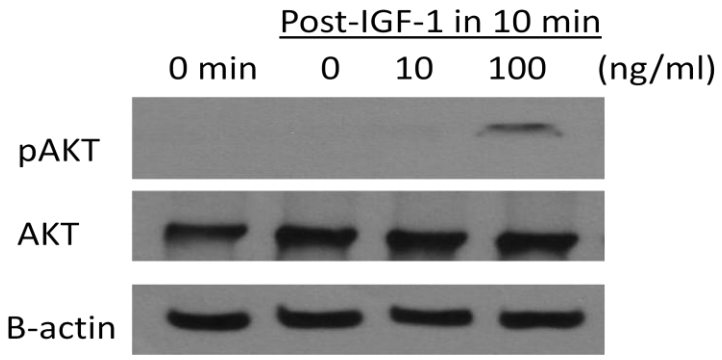
Figure 3.3 Continued.

3.3.4 Effect of IGF-1 and Zyflamend® on pAKT, IGF-1R and androgen receptor expression

To investigate whether IGF-1/IGF-1R signaling regulates androgen receptor expression and/or nuclear translocation via pAKT, CWR22Rv1 cells were treated with IGF-1 (10 and 100 ng /ml) for 10 min to stimulate AKT phosphorylation (Figure 3.4 A). To determine if these effects influenced the expression of IGF-1R and androgen receptor in the presence or absence of Zyflamend®, cells were pre-treated with IGF-1 (100 ng/ml) for 10 min, followed by treatment of ±Zyflamend® for up to 24 hr. As demonstrated previously, pre-treatment of cells with of IGF-1 increased pAKT expression, with no effect on IGF-1R and androgen receptor expression. Zyflamend®, on the other hand, reduced IGR-1R receptor expression (as reported previously), but increased phosphorylation levels at serine 473 on AKT were not associated with an induction ofandrogen receptor expression. These results question the link between pAKT and androgen receptor expression (Figure 3.4B).

To confirm that IGF-1/IGF-1R signaling was not influencing androgen receptor expression and nuclear translocation, cells were pretreated in the absence or presence of IGF-1 (100 ng/ml, 10 min) followed by Zyflamend® (200 ng/ml) for 24 hr and the levels of androgen receptor (cytosol and nucleus) were determined via immuno-fluorescent staining (Figure 3.4 C). Cellular and nuclear levels of androgen receptor were unaffected by preincubating cells with IGF-1, suggesting that IGF-1 signaling did not affect nuclear translocation. On the other hand, Zyflamend® reduced cellular and nuclear levels of androgen receptor independent of IGF-1.

A



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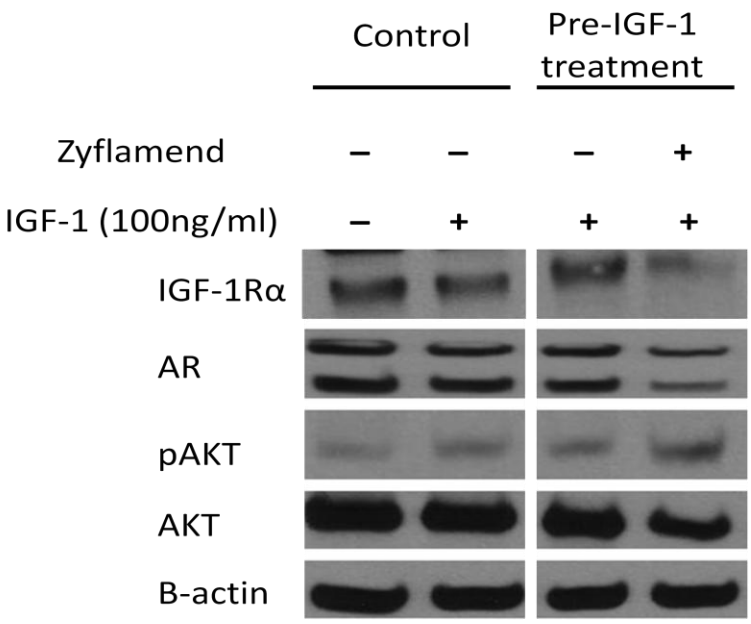


Figure 3.4 Effect of IGF-1 and Zyflamend® on pAKT, IGF-1R and androgen receptor (AR) expression. A. Effect of IGF-1 (10 and 100 ng/ml) on expression levels of pAKT. B. Effect of IGF-1 (pretreatment of 100 ng/ml for 10 min) in the presence or absence of Zyflamend® (0-24 hr). IGF-1Rα, AR, AKT and phospho-ser473 AKT were valued using immunebloting. C. CWR22Rv1 cells were treated with Zyflamend for 24 hr prior to treatment with IGF-1 (100 ng/ml). Expression of AR was evaluated using immuno-fluorescent staining. Nuclear AR intensity was quantified with DAPI staining. Values are means±SEM (n=250/group) and considered significant at $p<0.05$ as compared to control.

C

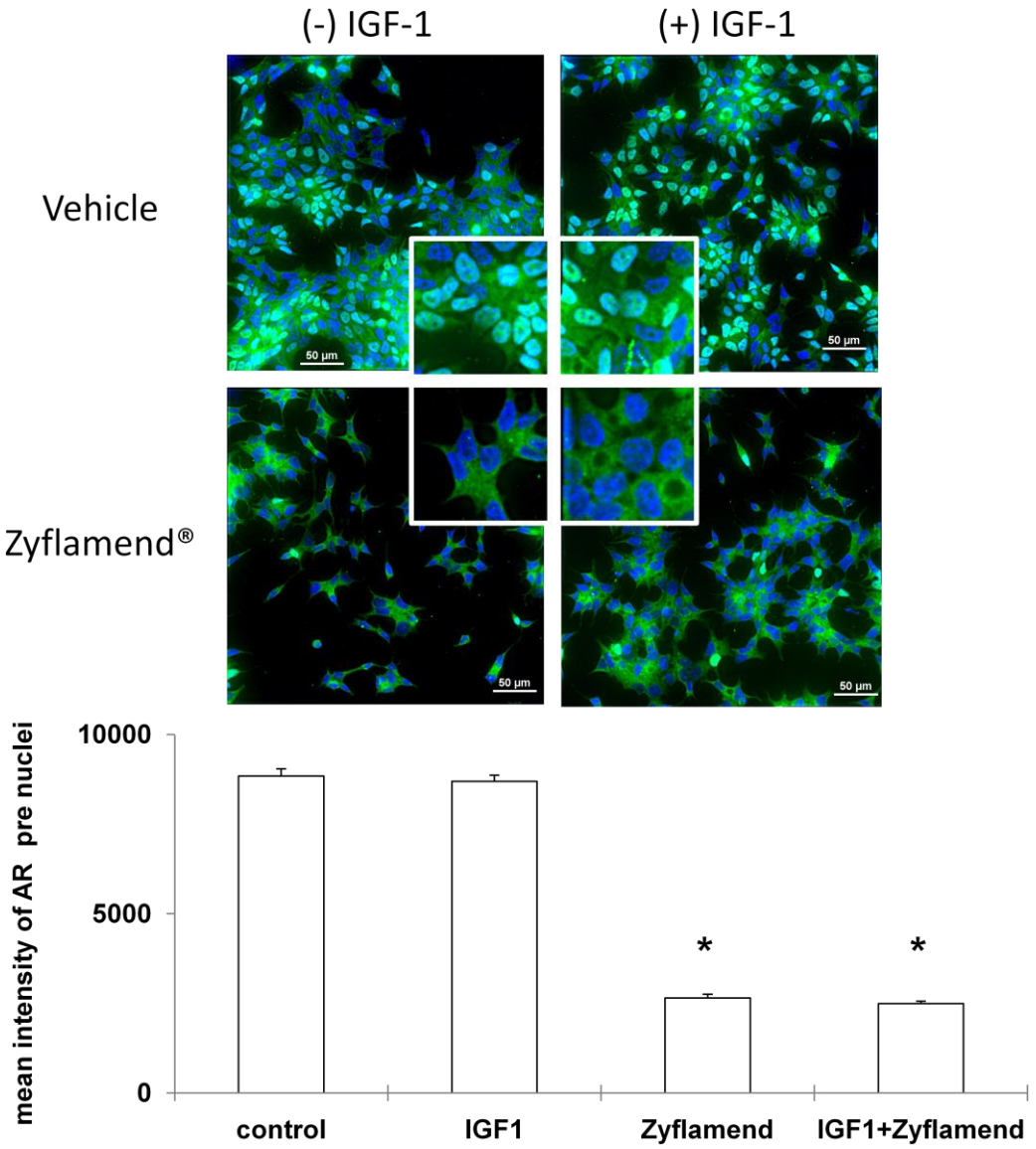


Figure 3.4 Continued.

3.3.5 Effect of overexpression of pAKT via adenovirus infection on androgen receptor expression and translocation

To investigate the effect of AKT phosphorylation on androgen receptor expression and nuclear translocation, overexpression of pAKT via myrAkt1 adenovirus was used. Increasing the levels of the adenovirus resulted in increased levels of AKT and pAKT, but had no effect on androgen receptor expression (Figure 3.5A). Zyflamend® treatment of adenovirus-infected cells inhibited androgen receptor expression and these results were not associated with pAKT levels. These results suggest that although Zyflamend® reduces the expression of androgen receptor (cellular and nuclear) and IGF-1R, these effects are not mediated via changes in pAKT.

3.3.6 Inhibition of PI3 kinase with wortmannin in the presence or absence of Zyflamend®

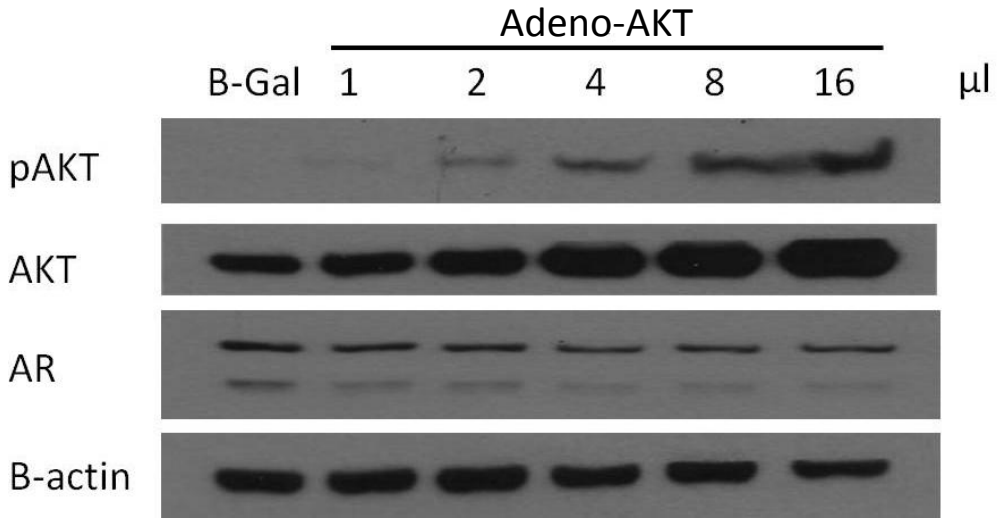
Wortmannin (1.0 μM), a PI3 kinase inhibitor, and Zyflamend® (200 ug/ml) inhibited cell proliferation in a time dependent manner. Wortmannin reduced cell growth by 57% after 96 hr treatment (Figure 3.6A). LY294002, another PI3 kinase inhibitor, showed the same reductions in cell growth over the time period (data not shown), indicating PI3K inhibition reduces CWR22Rv1 cell growth and these effects were similar to or greater than those observed with Zyflamend® (Figure 3.6B). . However, the inhibitory effect of wortmannin on the PI3 kinase pathway did not reduce androgen receptor expression (Figure 3.6 C).

3.3.7 Effects of Zyflamend® on biomarkers of apoptosis and proliferation

Zyflamend® (200 ug/ml) reduced the levels of pro-caspase 3 in a dose-dependent manner (Figure 3.7 A) and concomitantly increased caspase 3 activity (Figure 3.7 B). Cleavage of PARP, a downstream target of caspase 3, was increased in the presence of Zyflamend® (Figure 3.7 C).

Expression of the proliferative marker, Ki67 and the anti-apoptotic protein, Bcl-2, were inhibited by Zyflamend® treatment (Figure 3.7 C), suggesting Zyflamend® affects tumor cell number by coordinately increasing apoptosis and inhibiting cell proliferation.

A



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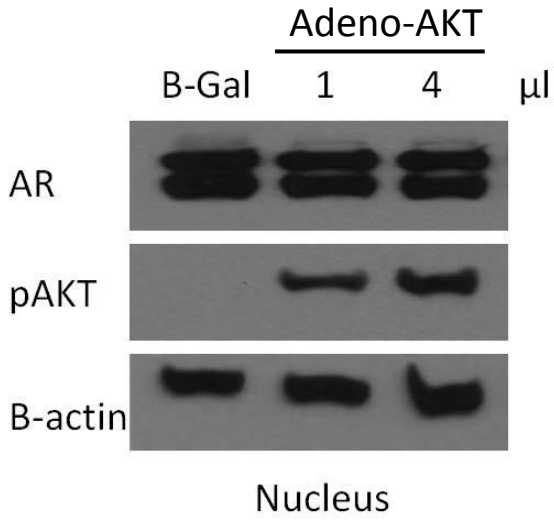
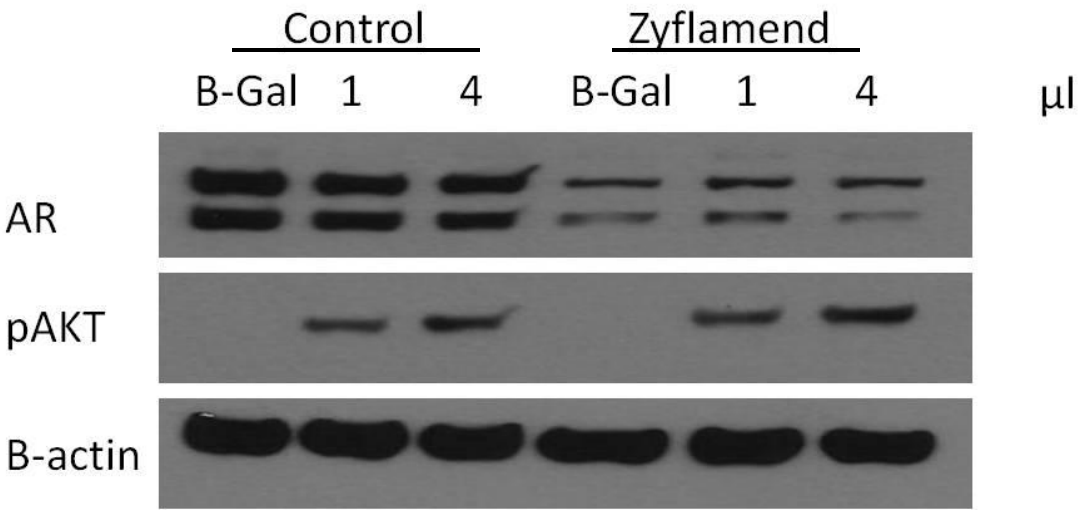


Figure 3.5 Androgen receptor expressions in CWR22Rv1 cells infected with the adenovirus myrAKT1 in the absence or presence of Zyflamend®. A. CWR22Rv1 cells were infected with adeno-myrAKT1 at uncreasing doses while β-Gal was used as the control. Total AKT, phospho-serine473-AKT, androgen receptor and β-actin were detected via western blot analysis. B. Androgen receptor levels in the nuclear fraction of CWR22Rv1 cells following infection with adeno-myrAKT1. C. Protein expression of androgen receptor and pAKT in CWR22Rv1 cells co-incubated with adeno-myrAKT1 and Zyflamend® (200 ug/ml).

C



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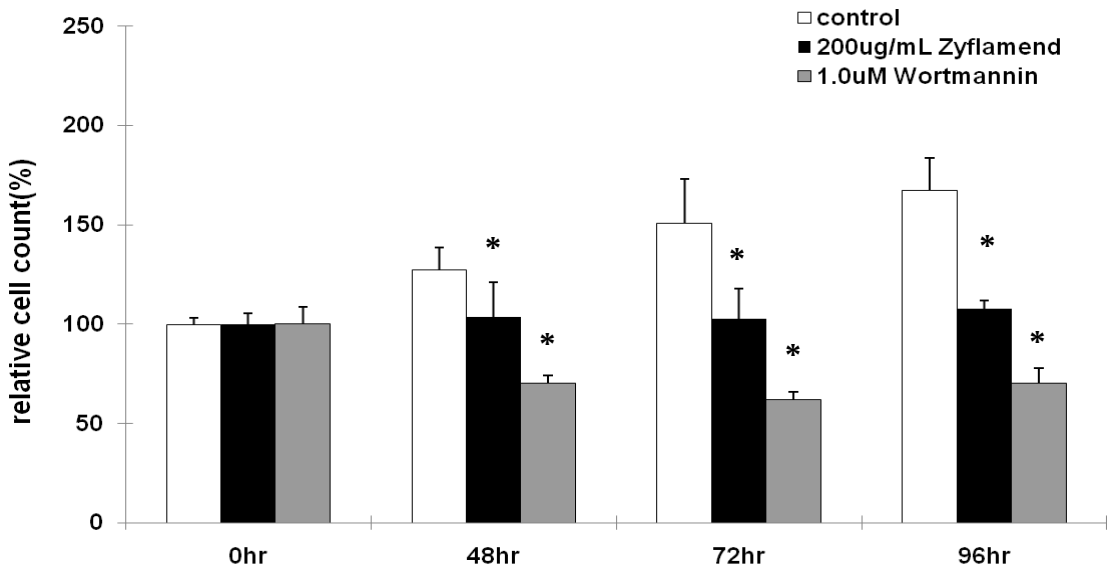

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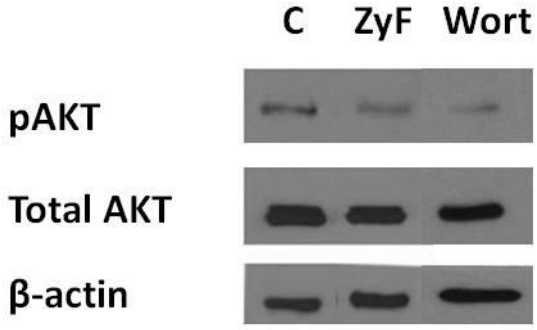

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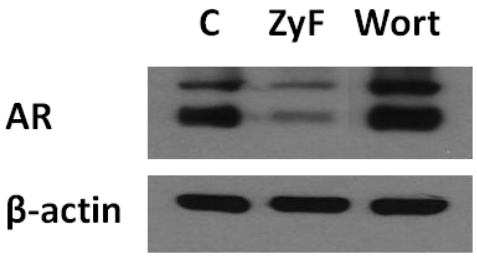

Figure 3.6 Effect of Zyflamend® (200 μg/ ml) and the PI3K inhibitor, wortmannin (1.0 μM), on cell proliferation and the expressions of pAKT and androgen receptor. Values are means±SEM (n=8/group) and considered significant at $p<0.05$ as compared to control. A. CWR22Rv1 cells were treated with Zyflamend® or the PI3K inhibitor, wortmannin and cell proliferation was evaluated from 0-96 hr. Values are mean±SEM (n=8/group) and groups are considered significant at $p<0.0$ 5. B and C. Protein expressions of pAKT and androgen receptor were determined after 48 hr treatment of Zyflamend® or wortmannin.

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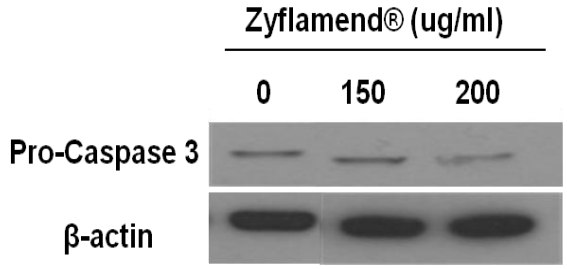

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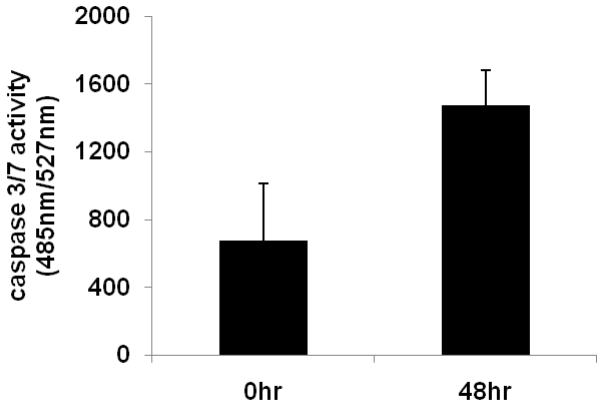

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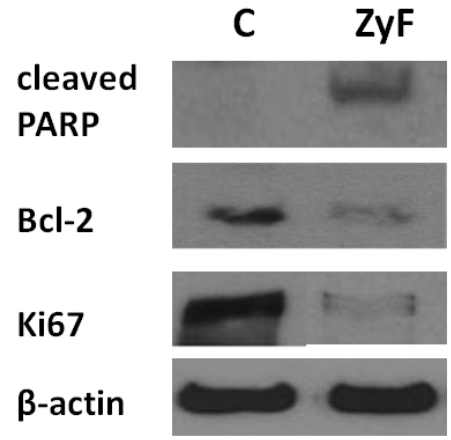

Figure 3.7 Effect of Zyflamend® on levels of pro-caspase-3,caspase 3/7 activity, PARP cleavage and expressions of Bcl-2 and Ki67 in CWR22Rv1 cells. A. Protein levels of pro-caspase 3 were determined after 24 hr treatment with Zyflamend® (0-200 ug/ml). B. Caspase3/7 activity after 24 hr treatment with Zyflamend® (200 ug/ml). Values are means±SEM (n=3/group) and considered significant at $p<0.05$. C. Protein levels of cleaved PARP, Ki67, and Bcl-2 were determined after Zyflamend® treatment (24 hr).

3.4. Discussion

Prostate cancer relapse following androgen deprivation therapy is of particular concern with more advanced forms of the disease, particularly in those patients with metastatic prostate cancer. Prostate cancer cells are dependent upon androgens for growth and androgen deprivation results in regression. However, the cancer inevitably relapses and up regulation of the androgen receptor is a huge concern in controlling castrate-resistant relapse (20), as such; amplification of androgen receptor signaling has been identified as a potential target (7, 17), where identification of compounds that can antagonize this pathway, with few side effects, is paramount.

One of these compounds could be Zyflamend®. We previously demonstrated that Zyflamend® can inhibit CWR22Rv1 cell proliferation via multiple mechanisms, most notably epigenetically through histone acetylation. This study confirmed that Zyflamend® inhibits proliferation by down-regulating the proliferative marker, Ki67 (consistent with our earlier findings that Zyflamend® inhibited BrdU incorporation), and modifying indices of apoptosis (increasing caspase 3 activity and PARP cleavage and reducing Bcl-2 expression).

Amplification of androgen receptor signaling includes overexpression of androgen receptor, induction of androgen receptor activity (8), or crosstalk with other receptor pathways such as IGF-1/IGF-1R (12, 21). Several mechanisms involving the activation of the IGF-1/IGF-1R/AKT signaling pathway on androgen receptor regulation have been proposed. One pathway involves the androgen receptor regulator Foxo1. IGF-1 activates IGF-1R and its downstream kinases, PI3K and AKT. Activated AKT (pAKT) translocates to the nucleus and phosphorylates Ser-253, Ser-316, and Thr-24 of Foxo1**.** Following phosphorylation, Foxo1

dissociates from the androgen receptor, allowing it to activate target genes that can promote cell growth (15). It has also been proposed that IGF-1 enhances nuclear translocation of androgen receptor and activation of co-regulators in the absence of androgens, possibly through the action of a variety of kinases, including AKT (17), and that transcriptional activity of androgen receptor can be regulated by AKT-mediated phosphorylation (22). On the other hand, there is contradictory evidence with regards to the role IGF-1/IGF-1R on androgen receptor signaling (23).

In this study, we observed that Zyflamend® concomitantly inhibited the expression of IGF-1R and androgen receptor and its nuclear translocation. Based on the reports in the literature, it seemed logical that these effects could be coordinately mediated via the PI3K/AKT pathway. When CWR22Rv1 cells were treated with the PI3 kinase inhibitors, wortmannin and LY294002 inhibited cell proliferation to the same extent as Zyflamend®; however, expression of androgen receptor was not changed.

In an effort to clarify the role of PI3K/AKT in this process, we observed that IGF-1 increased pAKT levels; however, it had no effect on androgen receptor levels, total or nuclear, when evaluated by immuno-fluorescent staining. In contrast, Zyflamend® treatment significantly reduced androgen receptor levels, independent of IGF-1. This regulation appears to be at the transcriptional and translational levels as protein stability was unaffected. Furthermore, Zyflamend® did not down-regulate pAKT despite inhibiting the expression of IGF-1R and AR; and in fact, in some instances pAKT levels appeared to be increased in the presence of Zyflamend®. It is possible that overexpression of pAKT could attenuate androgen receptor expression. Alternatively, androgen receptor turnover is

phosphorylation-dependent (involving AKT), targeting the androgen receptor for degradation by proteasomes (24). This hypothesis seems plausible given the fact that over-expression of pAKT (by infecting CWR22Rv1 cells with the adenovirus myrAKT) was inversely correlated with androgen receptor expression, but the levels of androgen receptor were unaffected by wortmannin despite reducing pAKT levels, the downstream target of PI3K. Similarly, pAKT failed to alter the levels of androgen receptor in androgen-independent LNCaP-AI cells (derived from androgen-dependent LNCaP cells), or following knocked down of AKT1 by siRNA (25).

Androgen receptor is critical to castrate-resistance/androgen-independence in more advanced forms of prostate cancer. Mutations in the androgen receptor are associated with castrate-resistance and appear to have gains of function that enhance the activation of androgen receptor (26-27). CWR22Rv1 cells have two isoforms and one mutant at 874 (substitution of tyrosine for histidine) (28), enhancing androgen receptor activity and resisting the effects of androgen deprivation. One is a 114-kDa protein and other one is a 75~80-kDa protein. The 114-kDa protein has a duplication of exon 3 encoding zinc finger domain of the androgen receptor DNA-binding site and the truncated protein lacks the ligand-binding domain (LBD), referred to as ARΔLBD, in COOH-terminus (18). These two isoforms don't exist in the parental cell line, CWR22 cells. These two isoforms are constitutively expressed, where the smaller isoform remains active in a hormone-deprived environment. It is possible that these changes could have influenced how pAKT regulates and IGF-1/IGF-1R influences androgen receptor signaling.

In summary, activation of androgen receptor signaling appears to be a critical step in

castrate-resistant relapse following hormone deprivation. It has been postulated that upregulation of IGF-1/IGF-1R signaling contributes to this process. Zyflamend® appears to antagonize these events by inhibiting the expression of IGF-1R and androgen receptor, resulting in significant reductions in nuclear localization. Whether these effects are mediated, in part, by PI3K/AKT signaling remains unclear.

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Chapter IV

Effects of a Combination of Herbal Extracts on Multiple Prostate Cancer Xenograft Models

Abstract

Prostate cancer (PrC) is the second deadliest cancer of males in the USA. Hormone ablation therapy (HAT), a major therapy for metastatic forms of the disease, results in tumor regression; unfortunately, residual cells re-grow in the absence of androgens (castrate-resistant), and the disease is invariably fatal within 2 years. Diet is not an appropriate primary therapy following metastasis or for refractory forms of the disease; however, diet may be effective as an adjuvant to HAT, potentially extending the latency period and delaying relapse and/or inhibiting refractory growth. Zyflamend® is a combination of extracts from multiple herbs, each with reported anti-cancer properties. There is evidence that this combination is far more effective than any herb in isolation. We and others have demonstrated that Zyflamend® can inhibit growth of various PrC cell lines, but no studies have investigated its potential use *in vivo* using a model of metastatic PrC. In this study, the inhibitory effect of Zyflamend® was examined using androgen-dependent and castrate resistant tumors in a mouse model that mimics the phases of this terminal stage of the disease. At human equivalent doses, Zyflamend® potentiated the effects of HAT in initiating tumor regression, where the time needed to reduce tumor size was almost cut in half and the response of the tumors to intervention was less variable. Zyflamend® reduced the expression of a number of biomarkers linked to PrC progression including pAKT, prostate specific antigen (PSA), histone deacetylases and androgen receptor. In summary, this is the first paper to report that a combination of herbal extracts when provided at human equivalent doses can potentiate the effects of hormone deprivation therapy on the regression of PrC and inhibit the growth of tumors derived from androgen-dependent and independent PrC cancer cells *in vivo.*

4.1 Introduction

Prostate cancer (PrC) is the most commonly diagnosed solid malignancy and has become the second leading cause of cancer-related deaths in men in most Western developed countries (1). Once tumors become metastatic, they are very difficult to treat, and prognosis is poor with a 5-year survival rate of 31% (2). For the most part, PrC is temporarily responsive to hormone deprivation therapy as prostate epithelial cells are dependent on androgens for growth.

Metastatic PrC can be divided into four distinct phases, all of which play an important role in determining the inevitable outcome of this disease (Figure 4.1). Phase I involves initial tumor growth following metastasis (i.e., bone). This is an androgen-dependent process characterized by continued increases in levels of prostate specific antigen (PSA). Phase II involves hormone ablation therapy (HAT; also referred to as hormone deprivation therapy) followed by tumor regression (characterized by reductions in PSA levels). Although more than 95% of patients will respond to HAT and enter into a stabilization phase (Phase III), most find their PrC will eventually relapse and become hormone refractory (Phase IV). This castrate-resistant phase of the disease is invariably fatal with treatments that delay death by only a few months. If we can delay tumor relapse, inhibit growth of androgen-dependent and refractory tumors, and understand some of the mechanisms related to disease progression, we might be able to increase survival time for this stage of the disease.

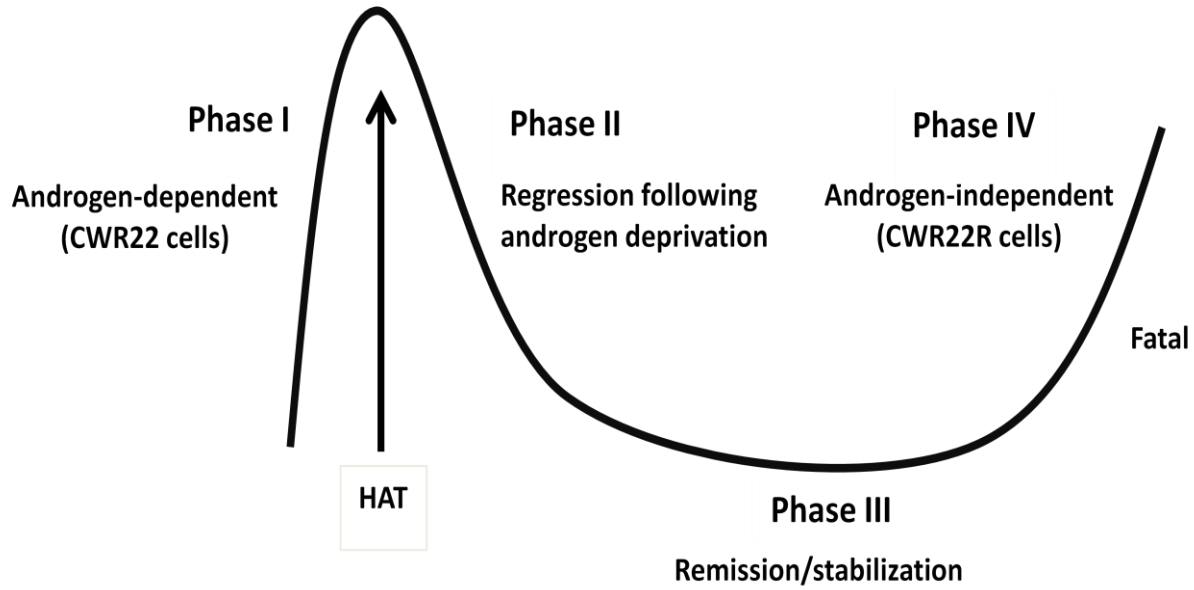

Figure 4.1 The theoretical phases of metastatic prostate cancer with androgen ablation therapy (HAT).

A preclinical experimental model mimicking this disease process has been developed using CWR22 cells. These androgen-dependent PrC tumor cells, when used in a xenograph mouse model, will grow into solid tumors in the presence of testosterone. These tumors will regress following testosterone ablation and eventually relapse into a refractory cell line (CWR22R cells) generating castrate-resistant tumors (3). CWR22Rv1 cells are a stable immortalized cell line that is derived from the CWR22R lineage. These cells are considered castrate-resistant because they can grow in the absence of androgens despite having a functional androgen receptor (4). Our previous work has demonstrated that diet can potentiate the effects of HAT and modify time-to-relapse, validating dietary intervention of the disease process using this model (5)

Currently, the use of medicinal herbs and their bioactive compounds have been shown to have beneficial effects on reducing models of PrC. Combinations of herbs and bioactive phytochemicals have been shown to be more effective than when used individually and it has been argued that these combinations are more clinically relevant because of their synergistic effects (6-7). Zyflamend® is one such combination. Zyflamend®, a polyherbal preparation derived from the extracts of ten common herbs, appears to provide clinical improvement to patients diagnosed with high grade prostatic intraepithelial neoplasia (HGPIN), a preneoplastic form of PrC (8). In addition, Zyflamend® has been shown to reduce proliferation in a variety of PrC cell lines by modulating genes that impact the cell cycle and apoptosis (9-11) at concentrations believed to be physiologically relevant where the individual herbs had no effect (Huang 2010).

Using castrate-resistant CWR22Rv1 cells we have demonstrated that Zyflamend® inhibits cell growth using physiologically relevant concentrations and these effects can be attributed to reductions in androgen receptor expression and increases in the cell cycle inhibitor p21 as regulated by histone acetylation (Huang 2010). However, no studies have used this experimental model (*in vivo*) to test the impact of bioactive herbals as the disease progresses from androgen-dependent to androgen-independent tumor growth. As such, this study will investigate the effects of Zyflamend® on androgen-dependent and castrate-resistant tumor growth *in vivo* and determine if there is preclinical value when used in conjunction with hormone ablation therapy at oral doses considered to be relevant to humans.

One of the animal models to study the natural responses of prostate cancer cells to hormone deprivation is the use of CWR22 PrC cells, derived from a primary tumor, which was established by Pretlow's group in 1993. CWR22 PrC cells are highly androgen dependent and cells regress after androgen removal and then relapse to transform an androgen-independent line, CWR22R. Relapse of tumor is in 30-50% of the animals within 3-10 months of castration (3). CW22RRv1 cells are castrate-resistant cells derived from relapsed CWR22 cells. CWR22Rv1 cells can survive in the absence of androgen but still response to androgen due to their isoforms of androgen recpetors (4) while these two isoforms don't exist in parental CWR22 cells. CWR22 cell line and its derivative, CWR22Rv1 cells, serve a good model to study the progression of prostate cancer to refractory.

Currently, the use of medicinal herbs and their bioactive compounds have been shown to have the beneficial effects on reducing prostate cancer progression and incidence. A combination of medicinal herbs is believed to have synergistic anticancer effects. In a CWR22Rv1 athymic nude mouse model, green tea polyphenols (GTPs) and EGCG postponed the tumor by 100% (54 days) and 33% (36 days) versus control (26 days), indicating the use of combination of phytochemicals inhibited prostate cancer cell growth, synergistically (12). These results showed that green tea polyphenols is more effective than single green tea bioactive compound, EGCG. Further, Zyflamend®, a polyherbal preparation derived from the extracts of ten common herbs, appears to provide clinical improvement to patients diagnosed with high grade prostatic intraepithelial neoplasia (HGPIN), a preneoplastic form of the disease (8). In addition, Zyflamend® has been shown to reduce proliferation in a variety of PrC cell lines by modulating genes that impact the cell cycle and apoptosis (9-11). To understand the effects of Zyflamend® on the progression of prostate cancer to refractory, CWR22 cells and CWR22Rv1 cells were

used and represented the different stages following hormone ablation therapy.

4.2 Materials and Methods

4.2.1 Supplement

Zyflamend® (New Chapter, Inc., Brattleboro, VT) is a combination of the extracts of ten herbs commonly used in food preparations (ginger, rosemary, turmeric, Chinese goldthread, holy basil, hu zhang, barberry, oregano, green tea and basil skullcap) (Table 4.1) (10) .

4.2.2 Cell lines

Human prostate cancer cell line, CWR22Rv1, was purchased from American Type Culture Collection (Rockville, MD). CWR22Rv1 cells are a castrate-resistant cell line that were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum under an atmosphere of 5% CO_2 at 37^{o}C. CWR22 cells (originally provided by Dr. Thomas Pretlow, Case Western Reserve University, Cleveland, OH) are an androgen-dependent human prostate cancer cell line that is propagated through donor mice (serial transplanted tumors) that are sensitive to androgen withdrawal, and thus, share greater similarity with primary prostatic tumors than immortalized cell lines (3).

Table 4.1 Compositions of individual herbal extract in Zyflamend®

Compounds	Percentage (%)[1]
Ginger	12.8
Rosemary	19.2
Turmeric	14.1
Chinese Goldthread	5.1
Holy Basil	12.8
Hu Zhang	10.2
Barberry	5.1
Oregano	5.1
Green Tea	12.8
Basil Skullcap	2.5
Total	99.7

[1]See reference 10

4.2.3 Animals

Five-week old castrated athymic male (nu/nu) nude mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were housed in wired cages with free access to water and fresh food and in a temperature-controlled room with 14 h periods of light and 10 h periods of darkness. The health of the animals was monitored daily. All animal manipulations were approved by the University of Tennessee Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1996, NRC).

4.2.4 Diets

The background diets used in this study were based on the composition of a typical Western diet with the following energy distribution: carbohydrate at 50%, fat at 34%, and protein at 16%. These diets were based on a modified version of the US17 diet (Research Diets, New Brunswick, NJ; diet # D05010801). The lipid composition was also designed to mimic that of the Western diet, ~15% monounsaturated fats, ~13% saturated fats and 6-7% polyunsaturated fatty acids (PUFA) where the PUFA distribution included 6% linoleic acid (LA), 0.7% alpha-linolenic acid (ALA), 0.09% long chain n-3 PUFA (eicosapentaenoic acid, EPA, plus docosahexaenoic acid, DHA), and 0.09% arachidonic acid (AA). The human equivalent doses of these PUFA for the murine diets were derived from human daily intakes of 15 g, 1.33 g, 200 mg and 200 mg for LA, ALA, EPA+DHA and AA, respectively, as shown in table 4.2. Human equivalent dose of Zyflamend®, which is ~2 one-gram capsules/d (13) or 0.32 % (g/100 g mouse diet), was used in the diet to maximize our ability to translate the results. When extrapolated based on differences in metabolic activity, the level of Zyflamend® is equivalent to a daily dose of 16 mg/day/mouse. We chose extrapolations using metabolic activity based on data evaluating interspecies comparisons with non-energy producing nutrients (14). Prior to all dietary experiments, the

animals were placed on the background diet for one week prior to being placed on background diets ±Zyflamend®.

Table 4.2 The dietary compositions in control diet and in Zyflamend® diet

Compositions	Control		Zyflamend®	
	gm	**kcal**	**gm**	**kcal**
Protein	17.4	15.8	17.4	15.8
CHO	54.7	49.7	54.7	49.7
Fat	16.8	34.4	16.8	34.4
Total		100		100
kcal/gm	4.4		4.4	
Ingredient				
Casien	150	600	150	600
L-Cystine	3	12	3	12
Corn starch	295	1180	295	1180
Maltodextin 10	75	300	75	300
Sucrose	100	400	100	400
Cellulose	50	0	50	0
Cocoa butter, deodorized	33	297	33	297
Linseed oil, RBD	3.95	35.55	3.95	35.55
Paml oil, bleached, deorderized	42	378	42	378
Safflower oil	23	207	23	207
Sunflower oil	21.6	194.4	21.6	194.4
Olive oil	0.79	7.11	0.79	7.11
Zyflamend (dry weight)	0	0	0.53	0
ARASCO (0.04g AA/ g)	8.75	78.75	8.75	78.75
DHASCO (0.04g DHA/ g)	8.75	78.75	8.75	78.75
Alpha linolenic acid, estyl ester	0	0	0	0
Oleic acid, estyl ester	5.9	53.1	5.9	53.1
t-BHQ	0.03	0	0.03	0
Mineral mix S10026	10	0	10	0
Calcium phoshate, dibasic	13	0	13	0
Calcium carboate	5.5	0	5.5	0
Potassium citrate	16.5	0	16.5	0
Vitamin mix V13401	10	40	10	40
Choline bitartrate	2	0	2	0
Alpha Vitamin E acetate	0.13	0	0.13	0
Total	877.9	3861.66	877.9	3861.66
	Fatty Acids (%) mole			
Total amount	100		100	
Total saturated fatty acid (SFA)	37.9		37.7	
Total monounsaturated fatty acid (MUFA)	41.4		41.4	
Total polyunsaturated fatty acid (PUFA)	20.7		20.9	
Total n-6 PUFA	18.6		18.8	
Total n-3PUFA	2.1		2.1	
Ratio PUFA/SFA	0.6		0.6	
Ratio PUFA/MUFA	0.5		0.5	
Ratio n-6 PUFA/ n-3 PUFA	8.9		8.8	

4.2.5 Fatty acid analysis for diets

Fatty acid analysis of the diets was performed as described previously (15) with the following modifications. Samples of powdered diets (100 mg), suspended in 0.9% saline with an internal control (1, 2-diheptadecanoyl-sn-glycerol-3-phosphorylcholine (Matreya LLC, Pleasant Gap, PA), were extracted with chloroform/methanol (1:2 v/v) followed by extraction (x2) with chloroform. Lipids were saponified with 0.5N NaOH/MeOH at 86°C for 15 minutes, followed by trans-methylation with BF3 in MEOH (Thermo Fisher Scientific, Morris Plains, NJ) at 86°C for 10 minutes. The preparation was neutralized with 0.7 N HCl in MeOH followed by the addition of saline. The fatty acid methyl esters were extracted with hexane and measured using a Hewlett Packard HP 5890 Series II Gas Chromatograph (Agilent Technologies, Inc. Santa Clara CA) with flame ionization detector and a DB23 fused silica capillary column (0.25 mm i.d. x 0.25 µm film; J&W Scientific, Folsom, CA), with hydrogen as the carrier gas. Fatty acids were identified when compared to standards (NuChek Prep, Elysian, MN).

4.2.6 Design of Xenograft Experiments

4.2.6.1 Androgen-dependent CWR22Rv1 tumor growth

CWR22 prostate cancer cells were grown in donor mice (five-week old castrated athymic male (nu/nu) nude mice) prior to transplantation into the experimental mice. Following growth of xenograft tumors, the tissues were homogenized with BD Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, CA) and subcutaneously inoculated into the right flank of the animals while a sustained-release testosterone pellet was implanted subcutaneously. One week after transplantation the animals were divided into two dietary groups, ±Zyflamend supplementation (10 mice/ group) and the growth of the tumors were monitored over the next 16

days (Figure 4.2A). Food intake, body weights, tumor size and wet weight, and serum PSA levels were measured at the end of the experiment.

4.2.6.2 Androgen-dependent CWR22 tumor growth following hormone deprivation

CWR22 prostate cancer cells were maintained in ten donor mice (five-week old castrated athymic male (nu/nu) nude mice) prior to transplantation into sixty experimental mice as described above. Sixty mice involved in the experiment were randomly divided into two groups, 30 for the control group and 30 for the Zyflamend® group. Tumors were allowed to grow in the presence of a slow-releasing testosterone pellet. When the tumors reached a size of 1.0 cm in diameter, the testosterone pellet was removed and mice were put on their respective diets (30 mice/group; two dietary groups, ±Zyflamend®) as shown in figure 4.2B. Additional identical experiment was repeated and ten animals were used in each group. This experiment was terminated at day 4 following testosterone pellet removal and xenograft tissues and blood samples were collected for analysis. The composition of the diets is described in Table 4.2. Regression of the tumors was monitored following the removal of testosterone after the animals were placed on diet. Food intake, body weights, tumor size and serum PSA levels were measured.

4.2.6.3 Castrate-resistant CWR22Rv1 tumor growth:

CWR22Rv1 cells (1×10^6) were suspended in serum-free RPMI 1640 medium mixed with BD Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, CA) at a ratio of 1:1 (v/v) and subcutaneously inoculated into the right flank of the animals. After tumor cells were injected, the animals were maintained on the background diet for 8 days to avoid dietary interference with initial tumor growth and cell survival, where upon 30 animals were randomly divided into two groups, 15 mice/group, ±Zyflamend®, for 12 days (Figure 4.2C).

Measurements of tumor size were initiated at day 0 and monitored every other day. Tumor volume was calculated using a formula of [$0.5 \times length \times width^2$] (16). Food intake and body weights were measured every day. Serum PSA levels and tumor wet weights were measured at the end of the study. Tumors did not grow in two animals in the control group and one animal in the Zyflamend® group, and thus, were excluded from the study.

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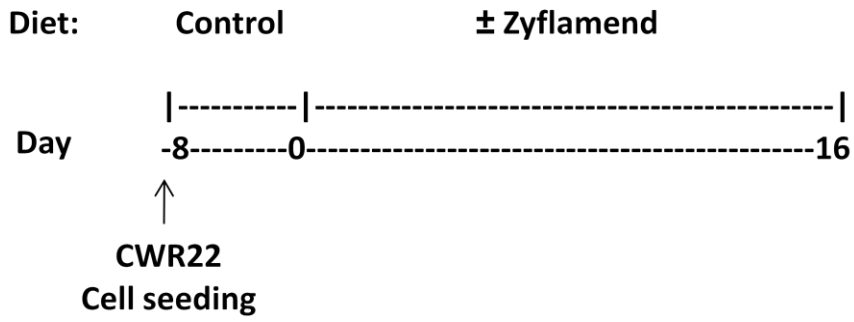

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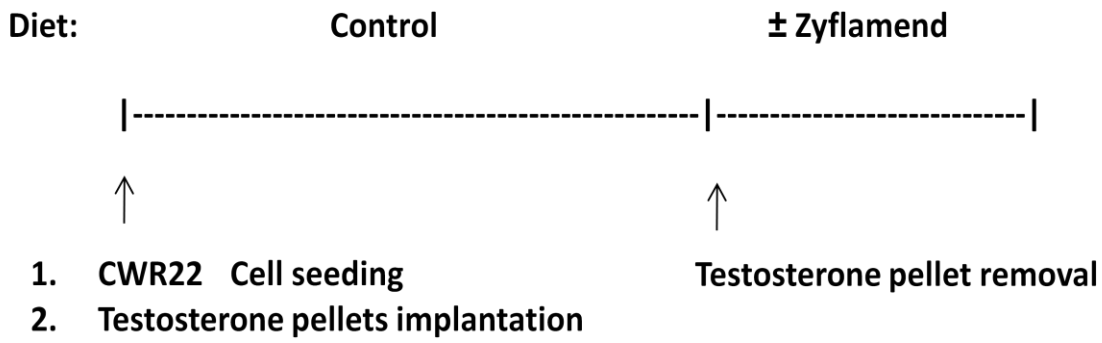

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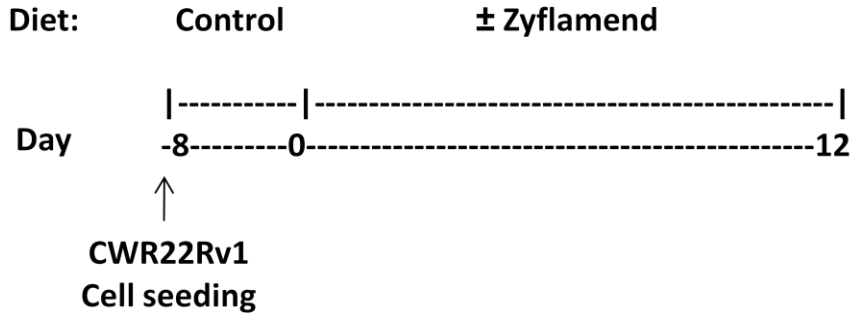

Figure 4.2 Dietary designs on androgen-dependent CWR22 xenograft model and castrate-resistant CWR22Rv1 xenograft model. A. CWR22 prostate cancer cells and testosterone pellets were transplanted into the athymic male (nu/nu) nude mice prior to switch to the experimental diet. Following transplantation (1 week), twenty mice were randomly placed into two groups (n=10/group), as indicated as day 0, and the study was terminated at day 16 when the length of tumors were between1.0 -1.5 cm. B. Dietary design using an androgen-dependent CWR22

xenograft model following testosterone pellets removal. CWR22 prostate cancer cells and testosterone pellets were transplanted into the athymic male (nu/nu) nude mice prior and the tumors were allowed to grow to a length of 1.0- 1.5 cm. The mice were then randomly placed into two dietary groups (n=30 /group), ±Zyflamend®. Additional identical experiment was repeated and ten animals were used in each group. This experiment was terminated at day 4. C. Dietary design using a castrate-resistant CWR22Rv1 xenograft model. CWR22Rv1 prostate cancer cells were transplanted into the athymic male (nu/nu) nude mice. After 8 days, the mice were randomly placed into two dietary groups ±Zyflamend® (n=10 /group). The study was terminated at day 12. .

4.2.7 RNA isolation and quantitative RT-PCR

Total RNA was isolated from CWR22 xenograft tissues using Trizol reagent (Invitrogen, Carlsbad, CA) followed by chloroform extraction. The aqueous phase was precipitated in 100% isopropanol and the pellet was washed in 75% ethanol prior to re-suspension in RNase-free water. Contaminating DNA was removed from RNA samples using Turbo DNA free™ kit (Ambion, Inc. Austin, TX) and then the concentration of total RNA was measured using NanoDrop 1000™ (Thermo Scientifc Wilmington, DE). Total RNA (2 μg) from each sample was mixed with MultiScribe™ Reverse Transcriptase, RNase Inhibitor, dNTP Mixture, random hexamers, RT buffer, $MgCl_2$ solution and incubated at 25℃ for 10 min, 48℃ for 30 min and 95℃ for 5 min to reverse transcribe to cDNA using TaqMan® reagent kit (Applied Biosystems, Carlsbad, CA). cDNA samples were used for quantitative RT-PCR (ABI Prism® 7300 Real-Time PCR System, Applied Biosystems, Carlsbad, CA). cDNA (0.7μl) was used as a template for qPCR amplification with primer sets (2.5 μM) of androgen receptor sense, 5'-CCTGGCTTCCGCAACTTACAC-3' and antisense 5'- GGACTTGTGCATGCGGTACTCA-3', and prostatic specific antigen sense, 5' - ACCAGAGGAGTTCTTGACCCCAAA-3' and antisense 5'- CCCCAGAATCACCCGAGCAG-3' were examined. Amplification was performed using a standard thermocycle program beginning with an initial temperature at 94°C for 1 min followed by 30 cycles of 94°C for 15 sec, 50°C for 30 sec and 72°C for 2 min. Each sample was examined in triplicate and the amounts of PCR product were normalized with 36B4, 5'-TGCATCAGTACCCCATTCTATCA-3' and 5'-AAGGTGTAATCCGTCTCCACAGA-3' as the internal control.

4.2.8 Western blotting

Xenograft tissues (20 mg) were lysed in the presence of cell lysis buffer (Cell Signaling Technology®, Inc. Danver, MA). Protein content of the lysates were quantified by BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Lysates (20 ug) were fractioned by 8~12% SDS-PAGE and transferred to a polyvinylidine diflouride (PVDF) membrane by electroblotting. The membranes were blocked using 5% nonfat dry milk in 0.1% tris buffered saline-Tween-20 (TBST) for 1 hour and were incubated in TBST containing primary antibodies overnight at 4°C. The membrane was incubated with anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) (Cell Signaling Technology®, Inc. Danver, MA). Protein expression was detected with a Pierce ECL Western Blotting detection system. Each membrane was exposed to Hyperfilm Film (GE Healthcare, Piscataway, NJ). Antibodies of AR, phosphor-AKT, AKT (Cell Signaling Technology®, Inc. Danver, MA), IGF-1R and COX2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used. β-actin (with anti-β-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)) was used as the control.

4.2.9 Serum PSA measurement

Serum PSA levels were measured using PSA ELISA kit (MP Biomedicals, Solon, OH) as per manufacturer's instructions. Briefly, blood was harvested and serum was isolated by centrifugation (13,000 x g at 4 °C for 20 min). PSA concentration was calculated based on readings at 450 nm using a SpectraCount microplate photometer (PerkinElmer Inc, Waltham, MA).

4.2.10 Statistic analysis

The results are presented as mean ± SEM and are analyzed by a two-way ANOVA when multiple comparisons were made. Differences were considered significant at $p<0.05$.

4.3 Result

4.3.1 Effects of hormone ablation on select biomarkers

The impact of hormone deprivation on select biomarkers of PrC was compared in a xenograph model using CWR22-derived tumors prior to and after the removal of a testosterone pellet in castrated athymic nude mice. Following testosterone removal, protein levels of androgen receptor, pAKT (the activated form of AKT), insulin-like growth factor -1 receptor (IGF-1R) and cyclooxygenase-2 (COX-2) were reduced by 85%, 60%, 46% and 49%, respectively (Figure 4.3 and Figure 4.4).

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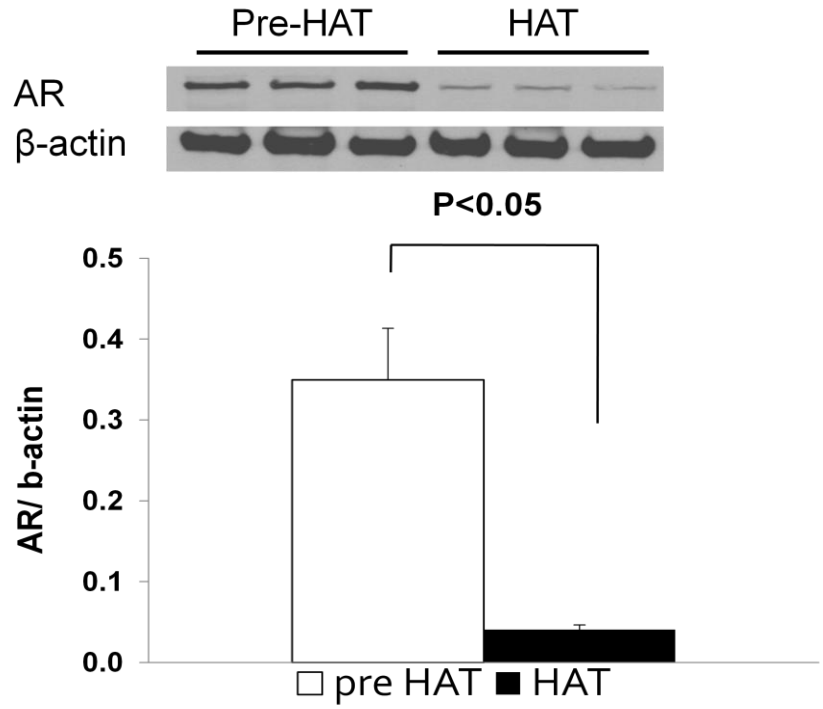

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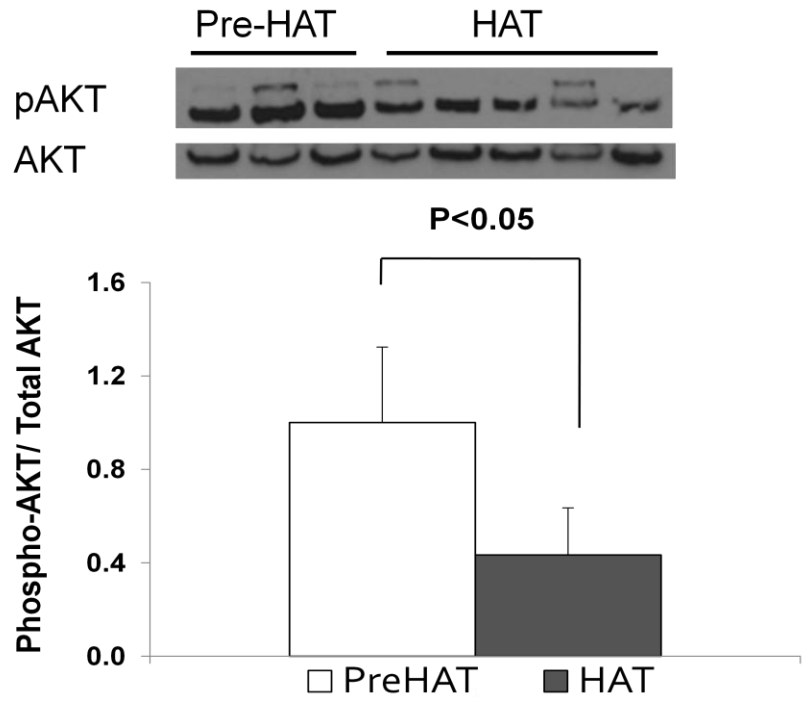

Figure 4.3 Protein expression of androgen receptor (AR) and phospho- AKT (pAKT) in CWR22-derived tumors prior to and after hormone deprivation. Bars are mean±SEM. HAT: hormone ablation therapy.

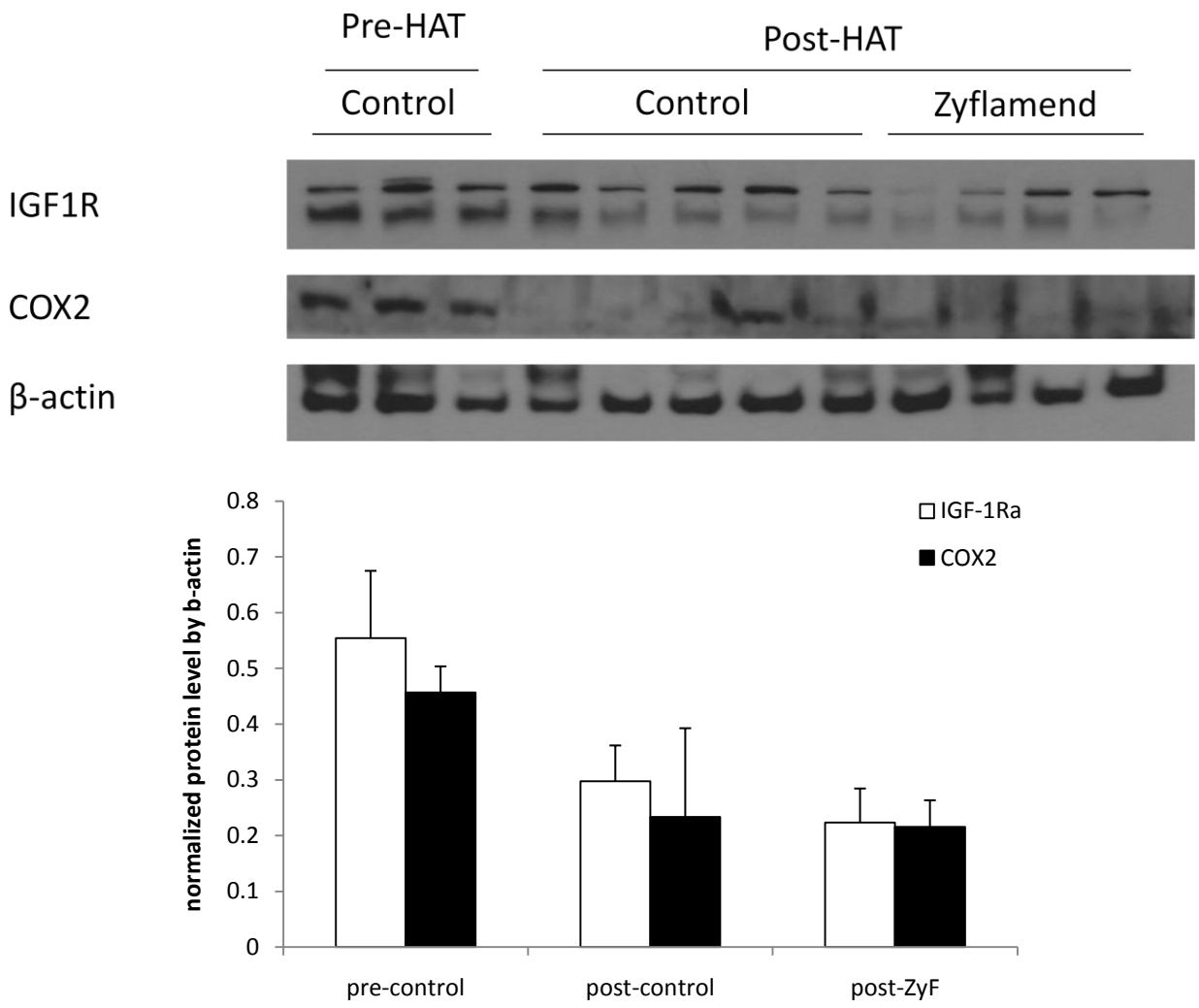

Figure 4.4 IGF-1R and COX2 expression (protein) from CWR22-derived tumors from mice on Control and Zyflamend®-supplemented diets prior to and after hormone deprivation. Bars are mean±SEM.

4.3.2 Effects of Zyflamend® on the growth of CWR22 xenograft tumors

CWR22 cells were transplanted into the flank of athymic nude mice in the presence of testosterone (a slow-releasing pellet inserted subcutaneously) and tumor growth was monitored in mice fed diets devoid of or containing Zyflamend®. After 16 days on diet, tumor size was significantly smaller (>50%) in those animals consuming Zyflamend® (Figure 4.5). There were no differences in body weights and food intake (Table 4.3). Circulating PSA levels were positively correlated with tumor size, where reductions (~40%) in tumor weight (wet weight) and PSA levels (ng/ml) mimicked the reductions (~50%) in tumor size. Expression of PSA mRNA was significantly reduced in tumors from mice fed Zyflamend® compared to mice fed the control diet; however, androgen receptor expression (protein and mRNA) was unaffected by diet (data not shown).

Table 4.3 Tumor sizes and biological indices from CWR22-derived tumors from mice on the Control and Zyflamend®-supplemented diets.

	Control diet	Zyflamend® diet	p-value
Relative tumor size (100%)	4.08±0.82	2.03±0.35	0.042
Tumor wet weight (g)	0.25±0.05	0.153±0.03	0.132
Food intake (g/d)	4.79±0.08	4.96±0.06	0.324
Body weight (g)	33.3±0.88	35.24±01.11	0.967
PSA (ng/ml)	90.69±17.27	55.76±18.34	0.186

Values are means±SEM.

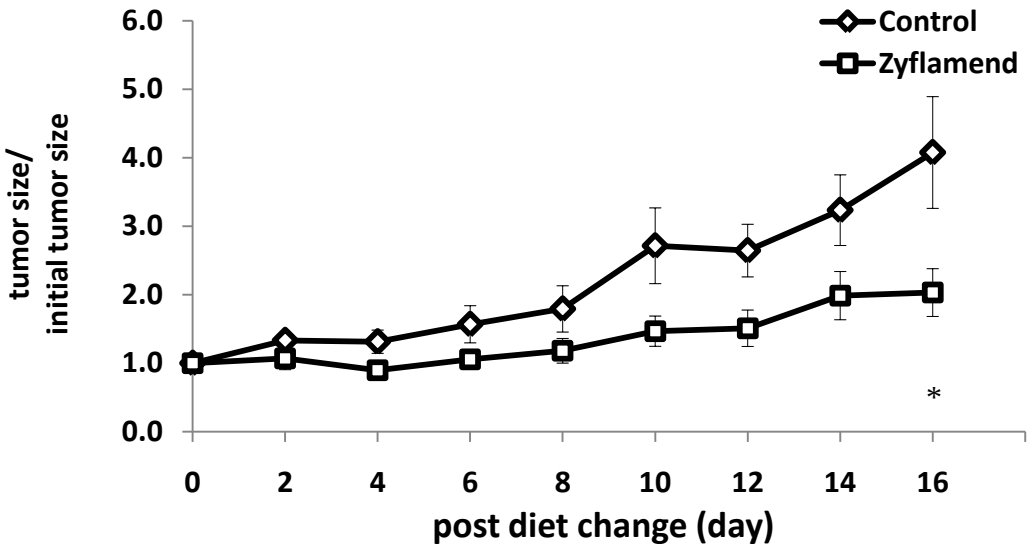

Figure 4.5 Tumor growth of CWR22cells inserted into the flank of nude (nu/nu) mice. The tumor size was measured and calculated using a formula of $0.5 \times \text{length} \times \text{width}^2$ and presented as a ratio of tumor size normalized to initial tumor size. Values are mean±SEM, n=10/group.

4.3.3 Effects of Zyflamend® on the growth of CWR22 xenograft tumors following hormone ablation

CWR22 cells were transplanted into the flank of athymic nude mice in the presence of testosterone and allowed to grow. When the tumors reached the appropriate diameter (1.0-1.5 cm) the testosterone pellet was removed, the animals were placed on their respective diets (±Zyflamend®) and tumor regression was monitored. Figure 4.6A represents the change in tumor size for each animal in each group following hormone deprivation. Within the first 4 days, tumors appeared to continue to grow in the control-fed animals prior to regression (Figure 4.6C), while tumor size in the Zyflamend®-fed group was reduced, after which time, tumor size continued to decline in both groups where Zyflamend® had a significantly greater effect compared to controls (within the first 20 days). The variability of response to hormone ablation was significantly smaller with Zyflamend® (Figure 4.6B). The length of time to reduce tumor size by an average of 50% in the Zyflamend® and control groups was 11days and 19 days, respectively ($p<0.05$) (Figure 4.6C). In addition, PSA levels (ng/ml) were correlated with tumor size (Figure 4.6D) and this was reflected in a reduction of PSA mRNA expression, but the changes in mRNA and protein levels of androgen receptor were not significant (Figure 4.7). Expression of IGF-1R was reduced post- versus pre-hormone ablation (Figure 4.4), but Zyflamend did not reduce IGF-1R expression further (Figure 4.4). Histone deacetylase 5 (HDAC 5) expression (mRNA) was lower in the Zyflamend® group compared to expression in tumors from the control group (Fig. 7). Food intake among dietary groups were not different (Table 4.4), but the animals in the Zyflamend® group lost less weight compared to those on the control diet ($p<0.05$) following hormone ablation.

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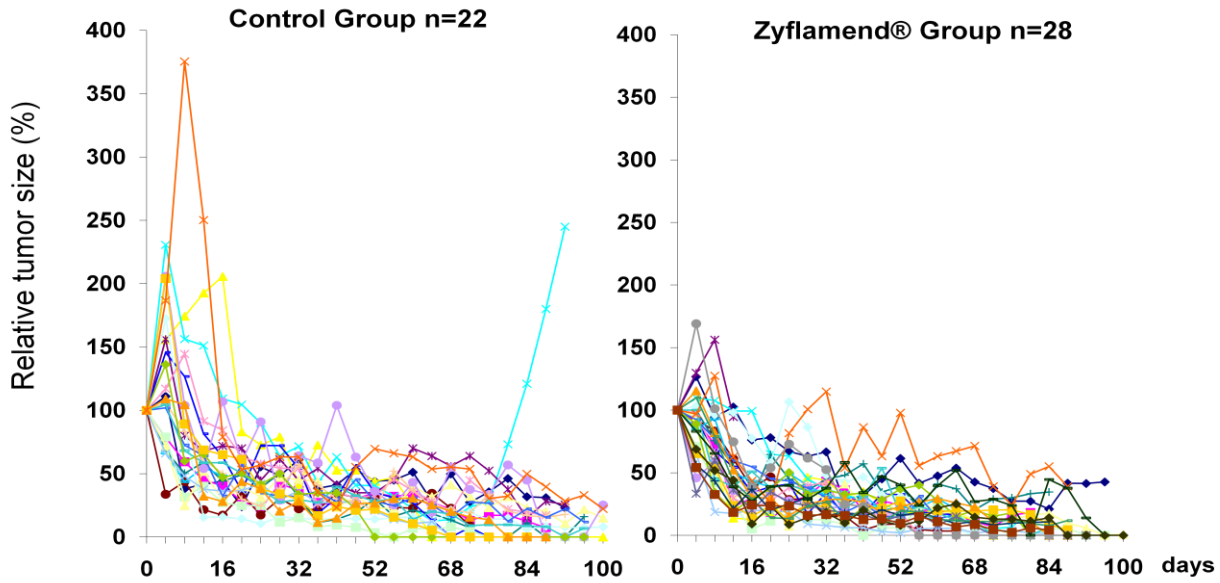

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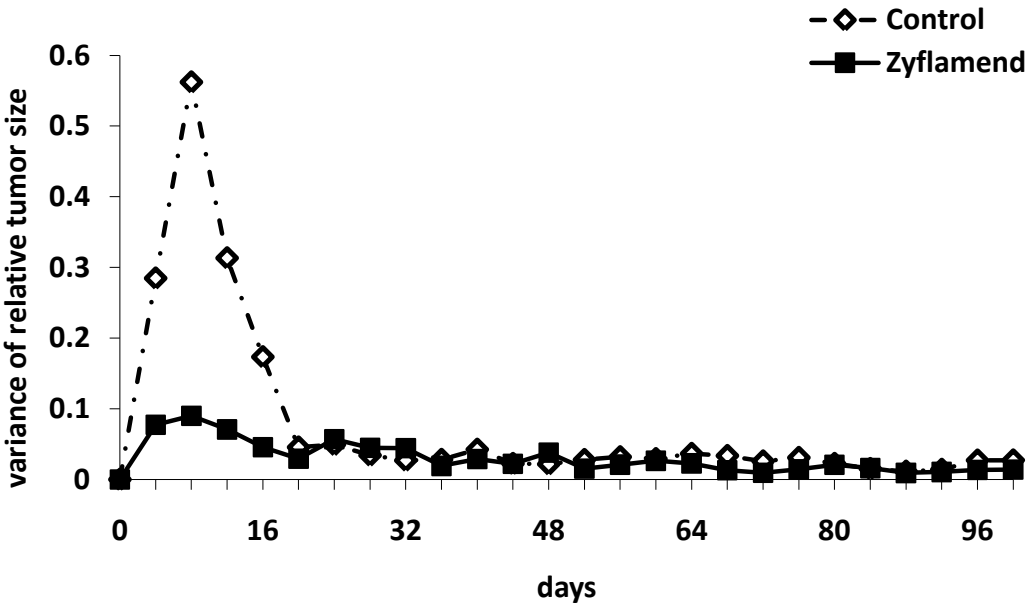
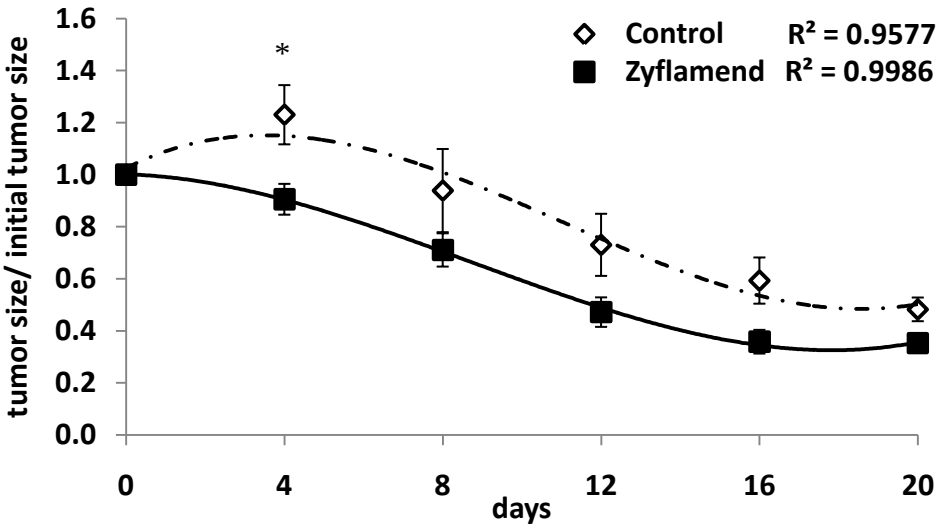


Figure 4.6 The impact of hormone deprivation on CWR22-derived tumors from mice on Control or Zyflamend®-supplemented diets. A. Relative tumor sizes in each individual animal over 100 days. B. Mean variances of relative tumor size for each dietary group over 100 days.. C. Changes in mean tumor volume for the first 20 days following hormone deprivation (mean±SEM). D. Mean serum PSA levels versus tumor sizes for each dietary group at day 4 after testosterone removal.

C



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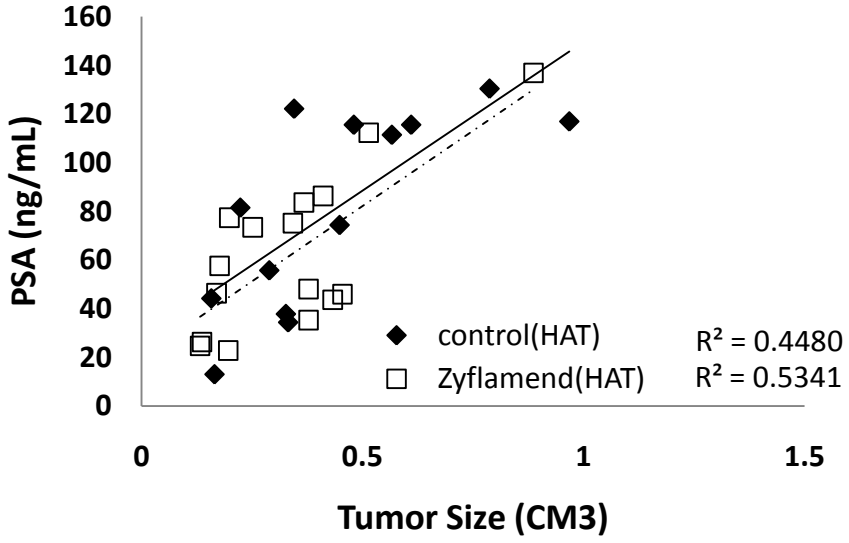

Figure 4.6 Continued.

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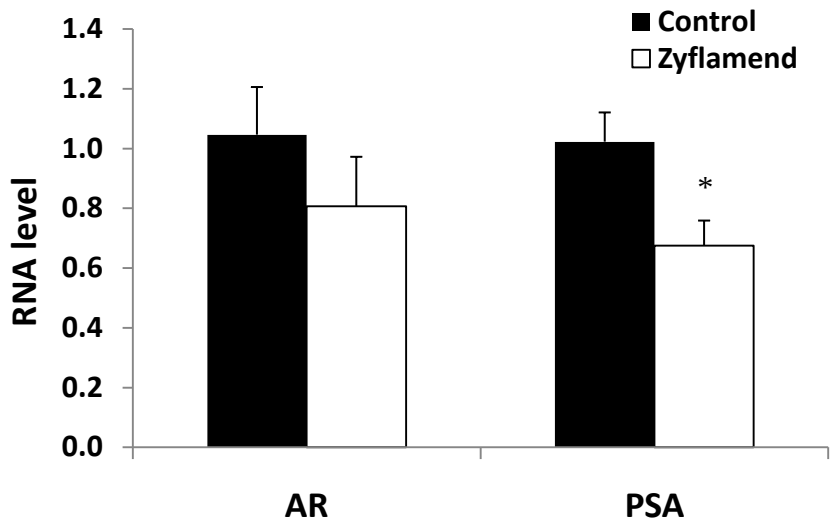

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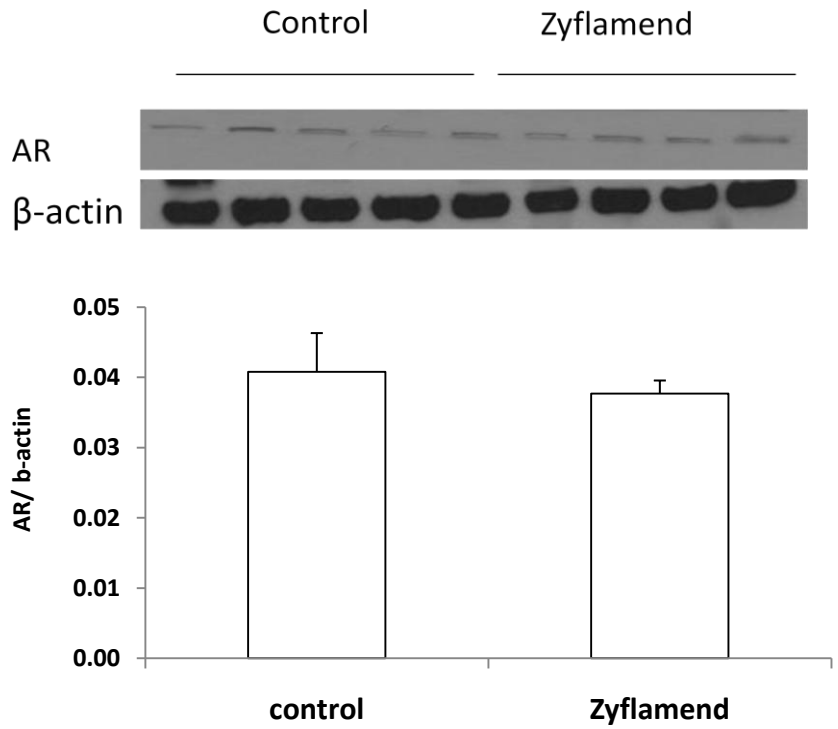


Figure 4.7 Expression of androgen receptor (AR) and PSA from CWR22-derived tumors from mice on diets from mice on Control or Zyflamend®-supplemented diets at day 4 following hormone deprivation. A. AR and PSA RNA expression levels of xenograft tissues were detected (n=12, mean±SEM). B. AR protein analysis of xenograft tissues were detected (mean±SEM, n=4-5/group).

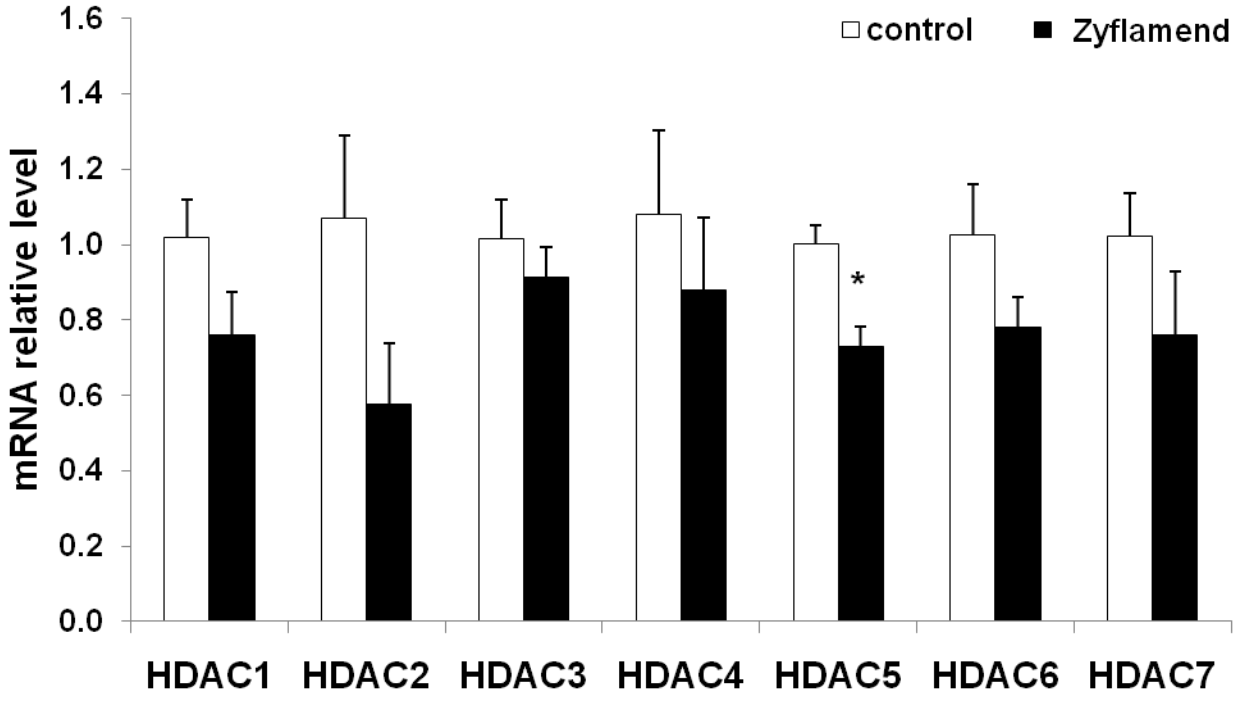


Figure 4.8 mRNA expression of class I and II histone deacetylases (HDACs) from CWR22-derived tumors from mice on diets from mice on Control or Zyflamend®-supplemented diets at day 4 following hormone deprivation. (bars are mean±SEM, n=5)

Table 4.4 Tumor sizes and biological indices from CWR22-derived tumors at day 4 compared to day 0 following testosterone pellets removal in mice fed Control or Zyflamend®-containing diets.

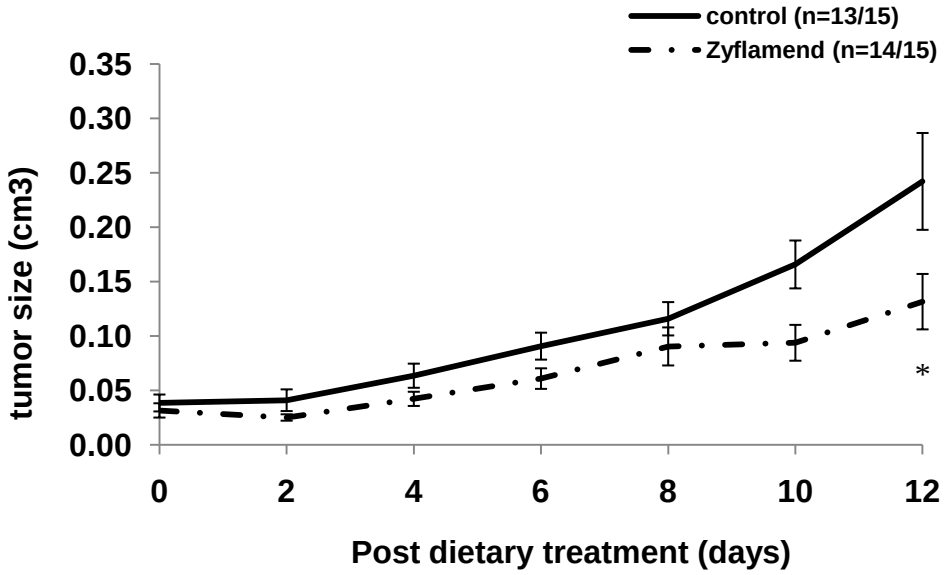
	Control diet	Zyflamend® diet	p-value
Relative tumor size compared to day 0 (100%)*	1.27±0.09	0.50±0.04	<0.001
Food intake (g/d)	6.0±0.46	6.1±0.44	0.638
Weight change (g)	-1.0±0.13	-0.5±0.15	0.001
PSA (ng/ml)	81.0±11.16	58.8±8.12	0.187

*Data was pooled from two individual experiments. Values are means±SEM.

4.3.4 Effect of Zyflamend® on androgen-independent growth of CWR22Rv1 xenograft tumor

Castrate-resistant CWR22Rv1 cells were transplanted into the flank of castrated athymic nude mice (in the absence of testosterone). After 8 days, the mice were switched to diets devoid of or containing Zyflamend® and tumor growth was monitored. After 12 days on their respective diets, tumor size was significantly smaller (46%) in those animals consuming Zyflamend® as tumor growth began to diverge within the first 2 days on the experimental diets (Figure 7.9A). Food intake and weight gain were not significantly different among dietary groups (Table 4.5). PSA levels (ng/ml) were low and could not be detected in 13/14 animals on the Zyflamend® diet, while almost half of the control animals (6/13) had measurable amounts of PSA (Table 4.5). Androgen receptor expression (protein) was significantly reduced in the xenograph tumors from mice fed Zyflamend® compared to tumors from mice fed the control diet (Figure 7.9B). HDAC 4 expression (protein) was lower in Zyflamend®-treated tumors compared to tumors from the control group (Figure 7.9C).

A



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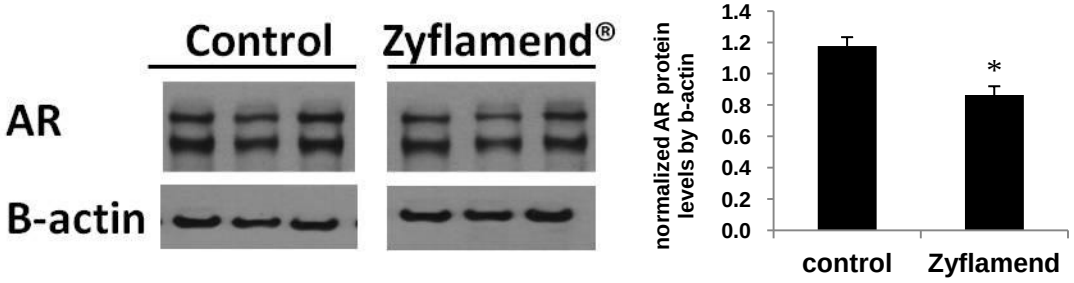

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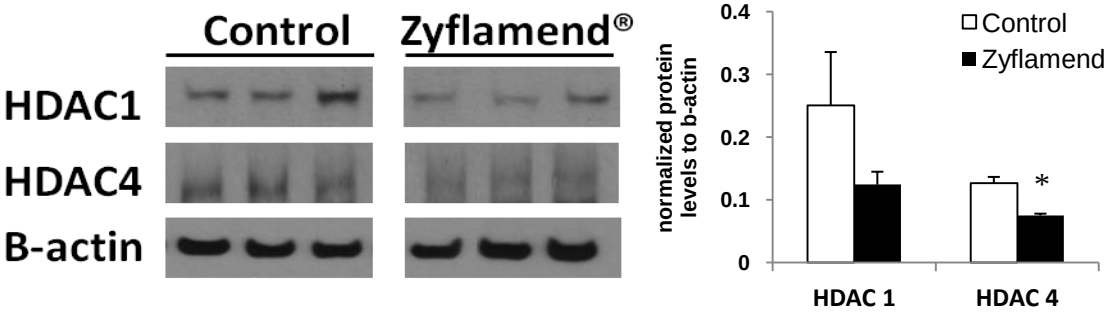

Figure 4.9 Growth of CWR22Rv1-derived tumors from mice on Control or Zyflamend®-supplemented diets. A. Tumor size was calculated using the formula $0.5 \times \text{length} \times \text{width}^2$. B. Androgen receptor expression (protein) in CWR22Rv1-derived tumors. C. Expression (protein) of histone deacetylases 1 and 4 (HDAC 1 & 4) from CWR22Rv1-derived tumors from mice on Control or Zyflamend®-supplemented diets.

Table 4.5 Tumor sizes and biological indices from CWR22Rv1-derived tumors from mice on the Control and Zyflamend®-supplemented diets.

	Control diet	Zyflamend® diet	p-value
Animal number	13	14	
Tumor size (cm^3)	0.24±0.04	0.13±0.03	0.033
Tumor wet weight (g)	0.18±0.02	0.12±0.03	0.074
Food intake (g/d)	2.95±0.05	3.10±0.03	0.098
Final body weight (g)	25.31±0.37	25.19±0.49	0.609
Weight gain (g)	2.28±0.19	2.91±0.27	0.062
PSA (ng/ml)	6/13*	1/14*	

*Number of detectable plasma PSA level in the group. Values are means±SEM.

4.4 Discussion

There are only a few preclinical experimental models of PrC and most of those do not address the progression of metastatic PrC. This paper reaffirms the proof of concept that metastatic PrC can be divided into four phases, where each phase may respond differently to therapeutic intervention with unique regulatory mechanisms (5). Furthermore, we propose that a mouse model of metastatic PrC may provide a unique opportunity to study the impact of diet as an adjuvant to standard therapies, in this case, hormone ablation.

Metastatic PrC travels to lung, liver and primarily bone. Following diagnosis, hormone deprivation therapy is commonly prescribed because prostate epithelial cells are dependent upon androgens for growth. This therapy results in tumor regression and stabilization for several years, after which time the residual cells appear to become refractory, resulting in castrate-resistant regrowth. Unfortunately, this relapse is invariably fatal within two years with few therapeutic strategies.

The experimental model used in this study follows a similar pattern. CWR22 cells are an androgen-dependent human prostate tumor cell line that mimics this process (3). When inserted subcutaneously in athymic nude mice, these cells grow into a solid tumor, and following androgen withdrawal the tumors shrink. However, relapse can occur within a year in the absence of androgens and the cells from the relapsed tumor, CWR22R, are classified as castrate resistant. An immortalized cell line derived from these relapsed cells, CWR22Rv1 cells, has been used as a model of castrate-resistant PrC because they produce human PSA, contain an active androgen receptor and can grow in the absence of androgens (17). This study explores the impact of diet in

a model of metastatic PrC in the presence or absence of testosterone and is an extension of our previous work with this cell model *in vitro* and dietary intervention on metastatic PrC (5).

Zyflamend® is derived from the extracts of ten different herbs and each has been reported to have medicinal properties *in vitro* (18-19, 7) and therapeutic value in humans. Individuals diagnosed with HGPIN are at increased risk for developing PrC (20). When this polyherbal preparation was used in a Phase I clinical trial with patients diagnosed with HGPIN, reductions in PSA and re-diagnosis to benign forms of prostate tissue were reported in 60% of the participants (8, 13). Based on this data, we investigated the effects of Zyflamend® in our model of castrate-resistant PrC. The oral dose of Zyflamend® for these *in vivo* studies was calculated to be ~600 mg/kg body weight (0.32% of diet, w/w). Allometric scaling for a human dose was based on the equivalent of ~two 1 g soft gel capsules of Zyflamend per day (based on energy for allometric scaling between species) (14). To give an idea how this dose may relate to individual doses of phytochemicals typically used, if one were to estimate the levels of curcumoids from Zyflamend®, it would be equivalent to ~2 mg/kg body weight per mouse per day (Zyflamend® contains ~0.35% curcumoids, w/w). These levels are well below typical levels of curcumin supplemented to the diets of rodents (200-1000 mg/kg body weight) (19). We used these conservative values in an effort to provide human relevance to our data.

We investigated the effects of Zyflamend® on androgen-dependent and castrate-resistant PrC tumor growth and explored its interaction with androgen deprivation. We focused our attention on several key biomarkers that have been related to metastatic PrC, *vis,* PSA and androgen receptor.

CWR22 cells represent those androgen-dependent metastatic tumor cells at the initial phase of tumor growth prior to hormone ablation treatment. Zyflamend® significantly inhibited tumor growth. This was somewhat surprising because dietary long chain n-3 polyunsaturated fatty acids (i.e., eicosapentaenoic acid, EPA), which are noted to be antitumorigenic, had no significant effect in this model at this phase of the disease process (5). We and others have shown that Zyflamend® inhibits growth of other androgen-dependent PrC cells *in vitro* (i.e., LNCaP cells) at doses considered physiologically relevant (review earlier chapters of this dissertation) (9). These previous effects were related to increases in biomarkers of apoptosis (caspase-3 activity and PARP cleavage) along with increased expression of the cell cycle inhibitor p21 (9). While we did not look at p21 expression in these tumors, we previously reported that Zyflamend® robustly increases p21 expression via epigenetic regulation in CWR22Rv1 cells.

Of interest is the ability to use diet to potentiate the impact of standard therapies, in this case, hormone derivation. The use of Zyflamend® in conjunction with the removal of testosterone potentiated tumor regression where the time needed to reduce tumor size was almost cut in half (11 days versus 19 days) and the response of the tumors to intervention was more immediate with Zyflamend® and less variable. The reduction in tumor size correlated with circulating PSA levels, an important biomarker for prostate epithelial cells and tumor growth. Although androgen receptor expression (mRNA) declined following hormone deprivation, we did not observe a significant reduction with dietary Zyflamend®, but PSA expression in those tumors was significantly reduced. The lack of a significant effect on androgen receptor expression with Zyflamend post-HAT could be due the fact that androgen receptor expression was down-regulated by 85% pre- to post-hormone ablation (in the absence of Zyflamend®).

Furthermore, pAKT expression, a reported regulator of androgen activation (21) was also significantly down-regulated (by ~60%) when testosterone was removed (independent of Zyflamend®).

This data and the data previously published with dietary EPA (5) are the first evidence to suggest that diet could be used in conjunction with hormone ablation to potentiate its effects. This could have an impact on potentially extending the subsequent latency phase prior to relapse, a key objective for increased survival (5). In humans, the latency period associated with hormone ablation therapy lasts approximately two years (22). Refractory growth is typically treated with chemotherapy in an effort to prolong survival by several months. It has been suggested that reactivation of androgen receptor in the absence of androgens may be one of the underlying mechanisms associated with this relapse (23).

Zyflamend® inhibited growth of CWR22Rv1-derived tumors by 46% within 12 days on diet. These cells represent a model of castrate-resistant tumor relapse. These results are consistent with data that shows Zyflamend® inhibits the growth of CWR22Rv1 cells *in vitro* (review earlier chapters of this dissertation), and with other androgen-independent PrC cells (i.e., PC3 cells) (11). The latter effect was associated with reductions in 12-lipoxygenase expression and phosphorylation of the cell cycle regulator retinoblastoma protein (Rb) (11).

The effects in the current study were associated with significant reductions in androgen receptor expression (protein). Similarly, using CWR22Rv1 cells *in vitro* androgen receptor expression and nuclear localization was significantly reduced following Zyflamend® treatment, further supporting our *in vivo* results. In addition to the reduction of androgen receptor, the inhibition effects of Zyflamend® were associated with a reduction in histone deacetylase activity.

Zyflamend® is a general HDAC inhibitor (class I and II) in CWR22Rv1 cells *in vitro* and here we show that Zyflamend® inhibits HDAC expression in xenograph tumors (Fig. 4.7 and 4.8). Inhibition of HDACs has been shown to inhibit androgen receptor and PSA expression in both hormone sensitive and insensitive PrC cells and thus could be likely mechanisms behind our effects (24).

In summary, we used a preclinical model of metastatic PrC by which the impact of diet can be investigated prior to or following hormone ablation therapy. Zyflamend®, a mixture of polyherbal extracts, inhibited androgen-dependent (CWR22 cells) and castrate-resistant tumor growth (CWR22Rv1 cells) *in vivo* using levels in the diet consistent with those recommended for humans. PSA levels correlated with tumor growth (or regression) and expression was reduced when Zyflamend® was added to the diet in all phases of the disease. Similarly, androgen receptor expression was inhibited following hormone deprivation and in castrate-resistant tumors, and these effects could be mediated by down-regulation of pAKT and HDAC expression. The mechanisms of action for Zyflamend® have been clearly elucidated *in vitro* (review earlier chapters of this dissertation) and are consistent with the results presented in this study.

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Summary and Conclusions

Advanced prostate cancer is aggressive and is the second leading cause of death from cancer in males in the United State. Malignant prostate cancer cells metastasize to adjacent and more distant tissues, such as bone. The characteristics of advanced prostate cancer can be outlined by four phases. The cells in the first phase are androgen-sensitive. Their growth is dependent upon the presence of androgens. Typically, the resultant tumors undergo regression following hormone ablation/deprivation therapy (Phase II), followed by an extended remission phase (Phase III). This remission phase usually lasts 3 to 5 years and eventually relapses (Phase IV). The relapsed cells grow in the absence of androgens and appear to be more aggressive with typical survival times limited to 1-2 years. Therefore, a critical barrier in treating advanced prostate cancer is finding effective treatments for castrate-resistant forms of the disease.

In this study, CWR22 cells and relapsed CWR22 cells (in the form of CWR22Rv1 cells) were chosen for the experiments because they represent those transitions associated with advanced prostate cancer. CWR22 cells only grow in the athymic nude mice supplied with testosterone and produce prostate specific antigen (PSA). After removal of testosterone, CWR22 cells regress and eventually relapse. These relapsed cells are referred to as CWR22R cells. They are castrate resistant, but still respond to androgens and can produce PSA. Similarly, prostate cancer cells in patients whose disease has relapsed following androgen ablation therapy express androgen receptors and produce PSA.

The use of herbs, botanicals and their bioactive components have been shown to be effective in many tumor cells lines *in vitro* and *in vivo* by inhibiting cell and tumor growth.

Zyflamend®, a polyherbal preparation derived from the extracts of ten herbs, appears to provide clinical improvement to patients diagnosed with high grade prostatic intraepithelial neoplasia (HGPIN), a preneoplastic form of the disease. Our findings suggest that Zyflamend® reduced cell proliferation in a variety of PrC cell lines, including RWPE-1 (representing premalignant cells), LNCaP (androgen-dependent), PC3 (androgen-dependent) and CWR22Rv1 (castrate-resistant) cells.

In the androgen-dependent phase of the disease (Phase I), Zyflamend® significantly inhibited growth of CWR22-derived tumors. Then, Zyflamend® potentiated the effects of hormone ablation (Phase II), reducing expression of PSA and HDAC5, important therapeutic targets. In an effort to address the issue of castrate-resistant relapse (Phase IV), CWR22Rv1 cells were used under *in vivo* conditions where mechanisms were extensively explored under in vitro conditions. Zyflamend® significantly inhibited CWR22Rv1-derived tumor growth. In vitro experiments demonstrated that the antitumorigenic effects of Zyflamend® were mediated, in part, by significantly enhancing the cell cycle inhibitors p21 and p27. These effects were mediated epigenetically via hyperacetylation of histones. Universal inhibition of histone deacetylases was responsible for these effects and these results were, in part, recapitulated *in vivo* (reductions in HDAC1 and 4 expressions).

Important to castrate-resistant relapse is the reactivation of the androgen receptor, possibly through the upregulation of IGF-1/IGF-1R signaling, and finding therapies that can target this process is a priority. Zyflamend® inhibited the expression of IGF-1R and IGF-1-stimulated cell proliferation. Levels of androgen receptor in CWR22Rv1 cells were reduced, including translocation to the nuclear fraction. These effects were originally believed to be

mediated by PI3K/AKT signaling through crosstalk between IGF-1R and androgen receptor; however, a definitive link could not be made.

The effects of Zyflamend are multifactoral and this is probably why it is so effective at the doses used. It inhibits tumor cell proliferation as evidenced by BrdU incorporation, inhibition of the expression of Ki67, fatty acid synthase and its upstream regulator SREBP-1c, and it increases markers of programmed cell death (increasing caspase 3 and PARP cleavage and reducing Bcl-2 expression). Furthermore, because epigenetic regulation of cancer is an environmentally driven process, finding therapeutic interventions that target acetylation and methylation processes are a priority. Zyflamend® appears to be a universal HDAC inhibitor. As such, the results *in vitro* and *in vivo* provide a logical rationale for the use of Zyflamend® in clinical trials because it appears to be effects at physiologically relevant concentrations, it has multiple mechanisms, in addition to its efficacy in multiple phases of a model of advanced prostate cancer.

Vita

E-Chu Huang was born in Taipei city, Taiwan. She was graduated from Tung-Hai University with a Bachelor of Science in Food Science and won the Phi Tau Phi Scholastic Honor. Two years later, she got her Master of Science in Food Science and Technology at National Taiwan University advised by Dr. Roch-Chui Yu. After graduating from National Taiwan University, she was employed as the research assistance in National Health Research Institute and directed by the formal President Dr. Chen-Wen Wu. The research was concentrated in the regulation of epithelial molecules on the metastasis in lung cancer. In 2005, she subsequently registered in the University of Tennessee as a Ph.D. student in the Department of Nutritional Science under Dr. Jay Whelan and she will receive her Ph.D. degree in 2010.