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AMPK Activation by Zyflamend: A novel pathway regulating metabolism and growth in prostate cancer

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To the Graduate Council:

I am submitting herewith a thesis written by Amber Frances MacDonald entitled "AMPK Activation by Zyflamend: A novel pathway regulating metabolism and growth in prostate cancer." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

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AMPK Activation by Zyflamend: A novel pathway regulating metabolism and growth in prostate cancer

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Abstract

Zyflamend, a select blend of 10 herbal extracts, effectively inhibits tumor growth using preclinical models of castrate-resistant prostate cancer (CR-PCa), mediated in part by activating AMPK, a master energy sensor of the cell. Currently, two predominant upstream kinases are known to phosphorylate/activate AMPK at Thr172: LKB1, a known tumor suppressor, and CaMKK2, a tumor promotor over-expressed in a number of cancers. Thus, the overall objective was to interrogate how Zyflamend activates AMPK and determine the roles of LKB1 and CaMKK2 in this activation, by targeting upstream mediators of both kinases ± Zyflamend using pharmacologic and molecular techniques in CWR22Rv1 (CR-PCa cell line) and HeLa (LKB1-null cell line) cells. Zyflamend-mediated activation of AMPK appears to be LKB1 dependent, while coordinately and negatively regulating CaMKK2 activity. Zyflamend failed to rescue the activation of AMPK in the presence of chemical and molecular inhibitors of LKB1 (radicicol & siLKB1), an effect not observed in the presence of inhibitors of CaMKK2 (STO-609, EGTA) in CWR22Rv1 cells. Using LKB1-null and catalytically dead LKB1-transfected HeLa cells that constitutively express CaMKK2, ionomycin (activator of CaMKK2) increased phosphorylation of AMPK, but Zyflamend had no effect. Zyflamend appears to inhibit CaMKK2 by DAPK-mediated phosphorylation at Ser511, an effect prevented by a DAPK inhibitor. Alternatively, Zyflamend increased pAMPK only in HeLa cells transfected with wild type LKB1. Zyflamend increased phosphorylation of PKCζ [Zeta], a known activator of LKB1, and inhibition of PKCζ reduced LKB1 phosphorylation. Using various constructs of HeLa cells, nuclear and cytosolic localization of LKB1 and PKCζ, respectively, were reversed following Zyflamend treatment, consistent with phosphorylation of AMPK by LKB1 in the cytosol. These results suggest that Zyflamend's activation of AMPK is mediated by LKB1, possibly via activation of PKCζ, but independent of CaMKK2. Moreover, Zyflamend inhibits CaMKK2 activity by a mechanism involving DAPK phosphorylation of CaMKK2 at Ser511.

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Introduction

Prostate cancer is the second leading cause of death for men in the United States [1]. While early stages of the disease are treatable, with 5-year survival rates near 100%, prognosis for advanced forms are less promising [2]. Initially, prostate cancer cells rely on androgens for growth, and chemically-mediated deprivation (hormone deprivation therapy) is a common therapy that results in cancer regression [3]. Relapse in the absence of androgens (castrate-resistant prostate cancer) is inevitable for most individuals and is associated with increased expression and activation of the androgen receptor, a major determinant in survival [4, 5]. Due to the poor prognosis of castrate-resistant prostate cancer, concomitant use of natural products to enhance effectiveness is being explored clinically and experimentally [4, 6-10].

Zyflamend (New Chapter, Inc. Brattleboro, VT) is a poly-herbal supplement derived from the extracts of ten different herbs: rosemary, turmeric, holy basil, ginger, green tea, hu zhang, barberry, oregano, Chinese goldthread, and baikal skullcap. Most research using Zyflamend has focused its effects on a variety of cancer models, including oral [11], mammary [12], bone [13], pancreas [14, 15], skin [11, 16], colorectal [15], with an emphasis on prostate [6-9, 17-21], and its beneficial effects appear to be related to the synergy of action of its components [22]. The effects of Zyflamend and its mechanisms on prostate cancer has been reviewed elsewhere and can be summarized in Figure 1 [3]. Zyflamend inhibits signaling pathways of inflammation, affects cell survival by enhancing apoptotic and tumor suppressor genes, epigenetically modifies histones, down regulates the androgen receptor and influences the energetics of the cell. The latter pathways are critically important in cancer as rapidly dividing cells rely on the increased synthesis of macromolecules (lipids, proteins, nucleotides, etc) (as reviewed in [23]).

5'-adenosine monophosphate-activated protein kinase (AMPK) is a key regulator of energy in the cell and responds to deficits in adenosine triphosphate (ATP). The protein contains a catalytic subunit (α-subunit), and two regulatory subunits, β and γ-subunits. Under conditions of energy stress the following occurs, *(i)* increased levels of AMP or ADP bind to the γ-subunit causing allosteric activation of the protein (ATP is a competitive inhibitor), *(ii)* increased affinity for upstream kinases that target phosphorylation at Thr172 of the α-subunit (increasing catalytic activity >100 fold), and *(iii)* reduced affinity for phosphatases that are involved in dephosphorylation at Thr172 [24-26]. When activated, AMPK is instrumental in inhibiting anabolic pathways that consume ATP, such as lipogenesis and protein synthesis, and enhances catabolic pathways that generate ATP, such as fatty acid oxidation [23, 27].

Recently, it was determined that tumor suppressor properties of Zyflamend is associated with the activation of AMPK and its downstream signaling [10]. This involves inhibiting the mammalian target of rapamycin complex-1 (mTORC1), the expression of fatty acid synthase and its regulatory transcription factor (SREBP1c), and inhibiting the activity of acetyl CoA carboxylase (ACC), a key regulator of the lipogenic pathway. What is not known is how Zyflamend upregulates AMPK. Four kinases have been identified that activate AMPK at Thr172, liver kinases B1 (LKB1), calcium-calmodulin kinases kinase-2 (CaMKK2), transforming growth factor-β activated protein kinase-1 (TAK1) and mixed lineage kinase 3 (MLK3) [28-32]. LKB1 and CaMKK2 are important in a number of cancers, including castrate-resistant prostate cancer (as reviewed in [33]), while the involvement of TAK1 and MLK3 has yet to be determined. LKB1 responds to increases in AMP and ADP, while increases in intracellular calcium is needed for activation of CaMKK2 without requiring elevation in AMP or ADP.

Interestingly, while both LKB1 and CaMKK2 are involved in activating AMPK, their effects on cancer appear to be quite different. LKB1 has anticancer properties because its

mutation/deletion is associated with a variety of cancers [34]. CaMKK2, on the other hand, is overexpressed in a number of cancers, including castrate-resistant prostate cancer [35-37]. Therefore, the overall objective of this research was to interrogate how Zyflamend activates AMPK in a model of castrate-resistant prostate cancer and the roles LKB1 and CaMKK2 play in that activation.

Chapter I of this thesis begins with an overview of PCa, Zyflamend's role against PCa, AMPK, and upstream regulators of AMPK (LKB1 and CaMKK2). In addition, Chapter II investigates Zyflamend's regulation of AMPK against PCa by determining the roles of LKB1 and CaMKK2.

Chapter I

Overview

Abstract

Prostate cancer (PCa) is the third most deadly cancer in men of the United States. Because PCa growth is androgen dependent, a common choice of treatment is androgen deprivation therapy (ADT). However, most tumors eventually become castrate-resistant PCa (CRPC), despite hormone ablation. This aggressive form of PCa can metastasize to other tissues (i.e. bone and lung), making the disease fatal within months. Therefore, it is critical to investigate effective treatments and mechanisms that inhibits CRPC growth. Zyflamend, a select blend of 10 herbal extracts, has been shown to inhibit CRPC *in vitro* and *in vivo* at human equivalent doses. Zyflamend's tumor suppressive effects are partly due to the phosphorylation and activation of AMP-kinase (AMPK), a master energy sensor of the cell that restores homeostasis in response to metabolic stress. AMPK activation by Zyflamend inhibits lipogenesis and enhances mitochondrial fatty acid oxidation, depriving proliferating tumor cells of necessary fatty acids for the generation of new membranes. However, the mechanism of action whereby Zyflamend phosphorylates AMPK (pAMPK) is unknown. Currently, the literature has identified two predominant upstream kinases that phosphorylate the catalytic subunit of AMPK at Thr 172, thereby increasing activity: liver kinase B1 (LKB1) and calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2). Inactivation of LKB1 is associated with a variety of cancers, including reduced expression in PCa tissue and LKB1 knockdown promotes its proliferation. In contrast, CaMKK2 is overexpressed in a variety of cancers and has been identified as a therapeutic target and novel biomarker of PCa. Therefore, identifying a therapeutic adjuvant that activates the LKB1-AMPK pathway, but simultaneously inhibits CaMKK2 activity could have therapeutic benefits.

1.0 Prostate Cancer

Prostate cancer (PCa) is a chronic disease of men that begins when cells in the prostate grow uncontrollably [38]. It is the third most common and deadly cancer of men in the United States [1, 38]. In 2017, the American Cancer Society estimates 161,360 new cases and more than 26,000 deaths from PCa [1]. Although the five year survival rate of localized prostate cancer is 100%, once the tumor metastasizes, it is difficult to treat and 5-year survival rates drops to 28% [38].

Androgens and PCa

Most prostate cancers are hormone-sensitive and depend on androgens (i.e. testosterone and dihydrotestosterone) for growth [39]. These androgens bind to and activate the androgen receptor, a transcription factor that regulates genes necessary for cell growth. Because early development of PCa is dependent upon androgens, this stage is characterized as androgen-dependent PCa [39].

Because initial PCa growth is androgen dependent, a common treatment is androgen deprivation therapy (ADT) which includes blocking the production of androgens and by antagonizing androgen receptor signaling. This results in tumor regression where patients may enter a stabilization phase and live with the disease for several years. Although more than 95% of patients respond to ADT, the cancer eventually relapses and progresses from an androgen-dependent to an androgen-independent state. This advanced form is commonly referred to as castrate-resistant prostate cancer (CRPC).

Despite a lack of androgens, robust androgen receptor (AR) signaling is a key characteristic of CRPC, making the disease difficult to treat, and with metastatic PCa invariably fatal within one to two years[40]. Androgen receptor gene amplification, mutations in the AR that results in constitutive activity and over expression of AR co-regulatory proteins is commonly observed in

CRPCs, suggesting this mechanism is involved with developing androgen independence [5]. In addition, expression of nuclear receptors such as transcriptional intermediary factor 2 (TIF2) and steroid receptor coactivator (SCR1) were increased in CRPC in comparison with benign prostatic hyperplasia and androgen-dependent PCa [5].Together, these alterations in AR gene amplification, mutations and expression of AR co-regulatory proteins could, in part, explain a constitutively active AR in a hormone refractory environment.

Treatments

Although ADT is a common treatment in PCa, other treatments may include chemotherapy, radiation, and immunotherapy. However, PCa tumors can metastasize to other tissues, such as the bone and lung, making the disease difficult to treat [3]. Because of the unpromising prognosis of CRPC, it is critical to investigate effective and safe treatments that delay or attenuate CRPC growth. A growing interest is the use of natural products (i.e. food and herbs) that can augment the effectiveness of standard therapies [41].

1.1 AMPK: Mechanistic Target of Cancer

Multiple pathways are involved in cancer regulation, but special attention has focused on the role of cellular metabolism on tumorigenesis, as first proposed by Nobel laureate Otto Warburg nearly 100 years ago [42]. Rapidly proliferating tumor cells generally shift from a catabolic to a more anabolic state, synthesizing macromolecules needed to sustain cell survival. Therefore, it has become of interest to switch this metabolic state and deprive tumors of the necessary macromolecules required for proliferation. A proposed mechanism promoting this metabolic switch is the activation of AMP-activated protein kinase (AMPK), a major energy sensor of the cell that responds to adenosine triphosphate (ATP) deficiency (as reviewed by Jeon *et al.* (2016)) [23].

Upon activation, AMPK inhibits ATP consuming pathways by inhibiting synthesis of major macromolecules including the synthesis of fatty acids, proteins, cholesterol, and glucose via gluconeogenesis, thus preventing cells from building essential nutrients for cell growth (Figure 1) [27]. Concomitantly, AMPK restores cellular energy homeostasis by increasing fatty acid oxidation, glucose uptake and glycolysis, oxidative metabolism, and autophagy. Although AMPK acutely increases glycolysis, in the long term it promotes mitochondrial biogenesis and oxidative metabolism, antagonizing the Warburg effect [27]. In addition, AMPK inhibits signaling of mammalian target of rapamycin complex 1 (mTORC1), via phosphorylation of TSC2 and/or phosphorylation of its regulatory subunit, raptor. Down-regulation of mTORC1 prevents synthesis of proteins needed for rapid cell growth, such as hypoxia-inducible factor-1α (HIF-1α) [27].

Other tumor suppressive functions of AMPK includes regulation of cyclin-dependent kinases (CDK), important enzymes that control the cell cycle. AMPK increases protein expression of CDK inhibitors, such as p21 and p27, which arrest the cell cycle and induces apoptosis [27].

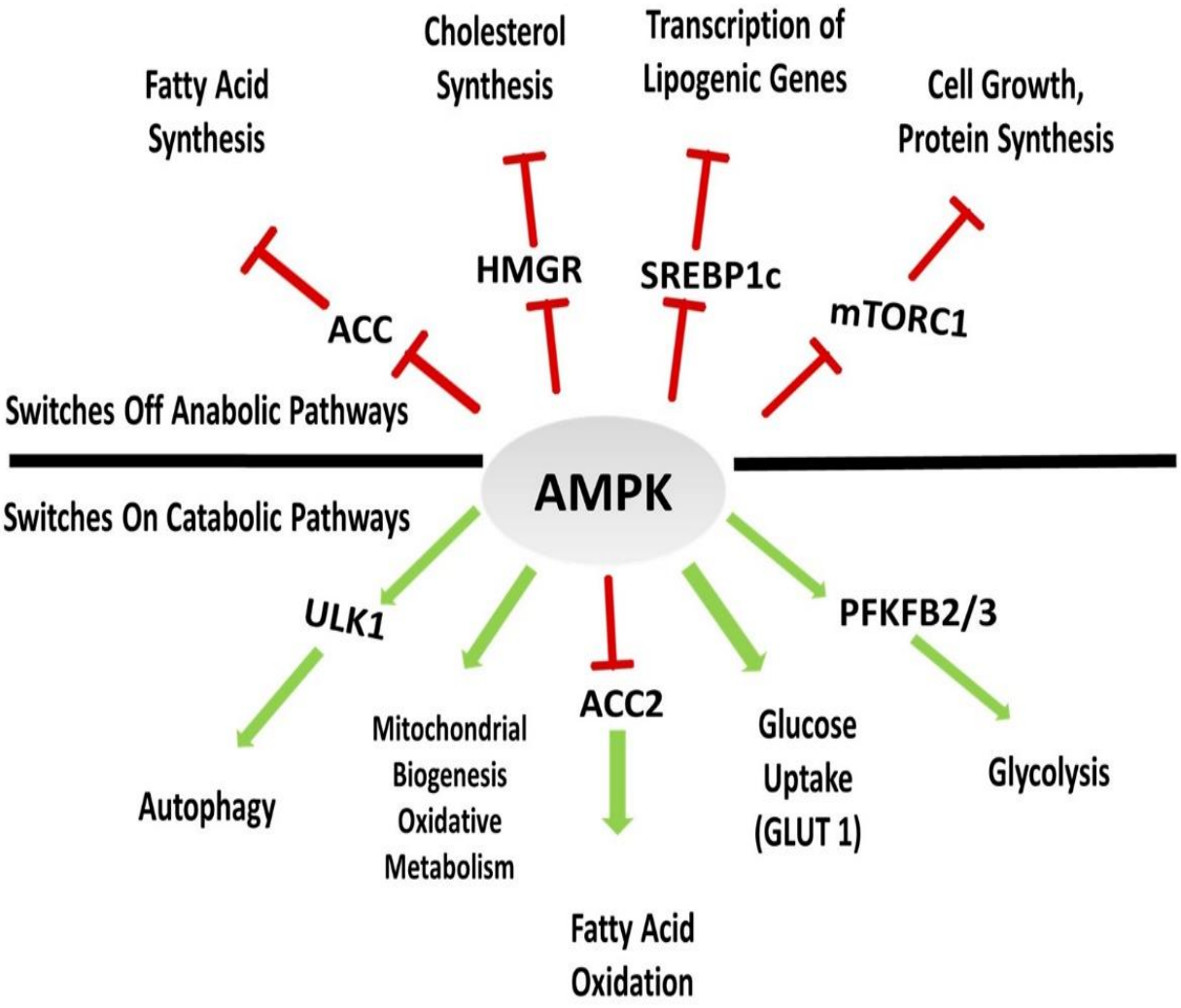

Figure 1. Summary of AMPK pathways. AMPK switches off anabolic pathways (shown in the top half) and switches on catabolic pathways (shown in the bottom half) [27].

Metformin, a pharmacological AMPK activator, is the most commonly prescribed anti-diabetic drug which lowers blood glucose by: (1) decreasing gluconeogenesis in the liver and (2) increasing translocation of GLUT4 to the plasma membrane to facilitate glucose entry into extra hepatic tissues [42]. Importantly, metformin lowers blood glucose independent of insulin, a growth factor that contributes to cancer risk and incidence [27]. These observations led to epidemiological studies which determined that diabetic patients taking metformin had a reduced incidence of cancer compared to patients taking other medications [27, 42]. In regards to PCa treatment, metformin was found to reduce prostate specific antigen (PSA) levels, which is a common biomarker of PCa progression [9]. A proposed mechanism of action whereby metformin stimulates AMPK activation is inhibition of the electron transport chain, thereby reducing mitochondrial ATP synthesis and increasing AMPK activation [42]. Overall, the activation of AMPK as a tumor suppressor protein, has become an important target in the treatment of cancer.

AMPK's Role in Prostate Cancer

Rapidly proliferating PCa cells are driven by an increased need for macromolecules mediated by lipogenesis, cholesterol synthesis, and protein synthesis, [43, 44] all of which are inhibited by AMPK activation. AMPK activation inhibits PCa proliferation in androgen-dependent and CRPC models, suggesting a pivotal role in both early and late stages of the disease [45]. In addition, AMPK activation was linked to decreased AR and PSA expression, and increased p21 levels [45]. Therefore, because AMPK can regulate multiple oncogenic pathways at once, it has become an attractive target to inhibit PCa growth [43-47].

Other studies suggest that AMPK's role in inhibiting PCa growth is controversial and that activation can either promote tumor suppressive or oncogenic activity [33]. New evidence indicates that different upstream stimuli of AMPK could determine whether activation signals

oncogenic or tumor-suppressive pathways [33]. Therefore, to inhibit PCa growth via AMPK, careful attention should be paid to its upstream regulators [33].

AMPK Structure and Activation

AMPK consists of a catalytic α subunit and regulatory β and γ subunits (Figure 2). Under conditions of cellular stress, such as nutrient deprivation or hypoxia, there is an increase of intracellular AMP:ATP ratio. With an increase in AMP, it binds to the γ subunit, resulting in a conformational change that permits phosphorylation and activation at Thr172 on the α subunit by select upstream kinases (Figure 2).

1.2 Upstream Regulators of AMPK

Currently, the literature has identified two predominant upstream kinases of AMPKα at Thr172: liver kinase B 1 (LKB1) [28] and calcium calmodulin dependent kinase kinase 2 (CaMKK2) [29].

LKB1's Role in Cancer

LKB1 is a serine/threonine kinase that is ubiquitously expressed in adult and fetal tissues [48]. It is considered a tumor suppressor protein because mutation/inactivation of its gene (*STK11*) is associated with a variety of cancers [34]. Mutation of LKB1 is the underlying cause of Peutz-Jeghers syndrome, a disease characterized by benign tumors in the gastrointestinal tract, with an increased risk of developing cancer, particularly colorectal and pancreatic cancers [49]. In pancreatic cancer cells, augmentation of LKB1 induced apoptosis modifies cancer cell survival *in vitro* [49]. LKB1 is mutated in ~30% of non-small-cell lung cancers (NSCLC),[50] making it the third most mutated gene in NSCLC,[49] and 20% of cervical carcinomas [42]. In breast carcinomas, silencing of LKB1 expression was associated with metastasis and shorter survival times, and *in vivo* studies showed LKB1 expression suppressed tumor growth and lung metastasis, suggesting LKB1 influences breast cancer progression [49]. In regards to PCa, LKB1

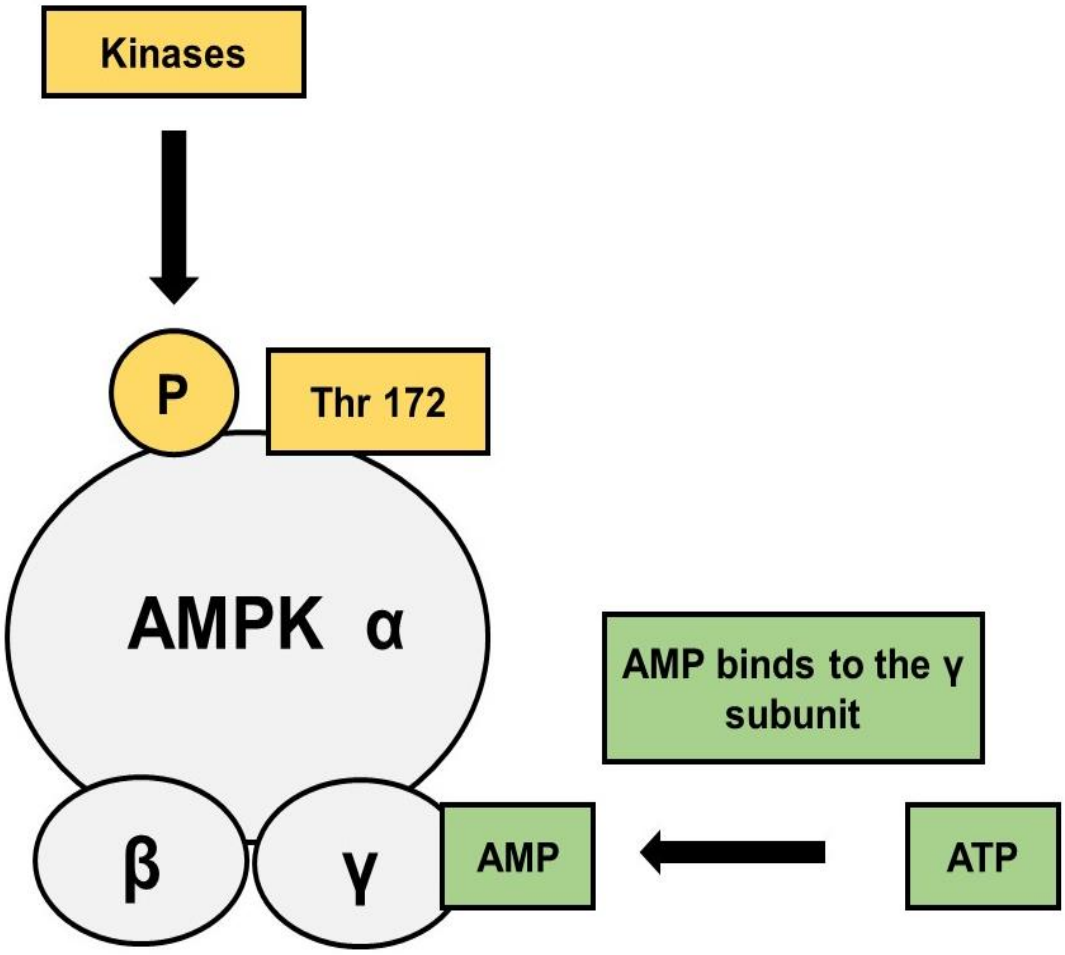

Figure 2. Structure and activation of AMPK. AMPK is a heterotrimeric protein consisting of a catalytic α subunit and regulatory β and γ subunits. Activation of AMPK requires binding of AMP to the γ subunit followed by phosphorylation at Thr 172.

expression is reported to be lower as compared to normal tissue [51, 52]. Similar results were observed in androgen-independent PCa cells (PC3 and DU145 cells) compared to normal prostate epithelial cells (RWPE-1), and LKB1 knockdown promoted their proliferation [51]. Consistent with these observations, in a clinical trial involving PCa patients, expression levels of LKB1 (mRNA and protein) were significantly lower in PCa tissue as compared to normal adjacent tissue and PSA levels, and survival times were inversely associated with LKB1 expression [52]. Overall, the expression and activation of LKB1 is important in multiple cancers.

LKB1 Structure and Activation

Human LKB1 has 433 amino acids and exists as two isoforms: LKB1 long form ($LKB1_L$) and LKB1 short form ($LKB1_S$) [53]. The N-terminus domain (residues 38-43) contains a signal that localizes LKB1 in the nucleus where it is inactive [49, 53]. LKB1 activation involves (*i*) phosphorylation and (*ii*) co-localization with two other proteins that results in translocation from the nucleus to the cytosol [54, 55].

Currently, there are six identified phosphorylation sites of LKB1: Thr 336, Thr 402, Ser 325, Thr 363, Ser 428, and Ser 399. Thr 336 and Thr 402 are auto-phosphorylation sites, while Ser 325, Thr 363, and Ser 428 can be phosphorylated by upstream kinases [56]. Ser 428 is only found in $LKB1_L$ and is reported to be essential for LKB1-mediated activation of AMPK [53]. However, Ser-399 may be a phosphorylation site of $LKB1_S$ that is analogous to Ser 428 of $LKB1_L$.[53] Although $LKB1_S$ can activate AMPK,[53, 57] it is primarily found in the testes [57]. Therefore, Ser 428 appears as a fundamental phosphorylation site required for LKB1-AMPK activation in PCa.

Following phosphorylation at Ser 428, LKB1 is bound by STRAD (STE20-Related Adaptor) and MO25 (Mouse protein 25) to form the active LKB1-STRAD-MO25 complex in the nucleus (Figure 3) [54, 55] (others describe this formation can occur in the cytosol) [48]. STRAD involvement increases LKB1 activity, while MO25 increases stabilization of the active complex (as reviewed in

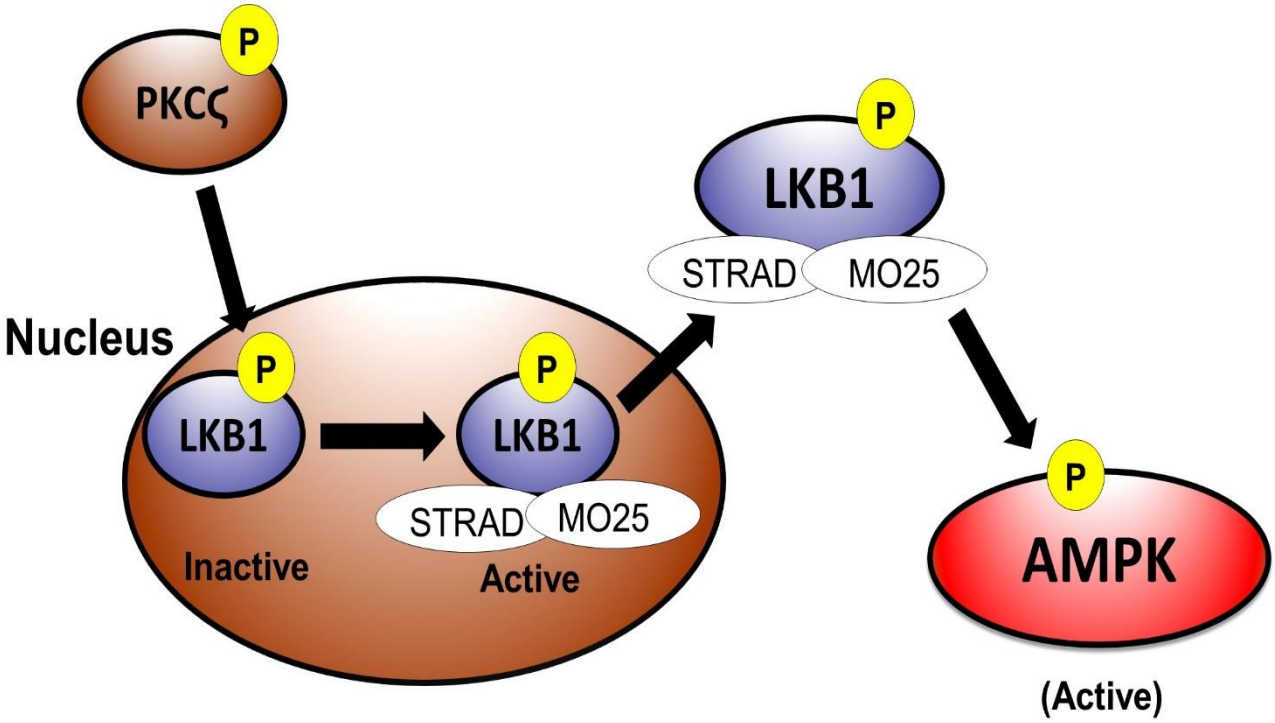

Figure 3. LKB1 activation and translocation. LKB1 is mostly found inactive in the nucleus where it can be phosphorylated by PKC-ζ and form an active complex with STRAD and MO25. The LKB1-STRAD-MO25 complex is then translocated from the nucleus into the cytosol where it can phosphorylate AMPK.

reference [58]). The active LKB1-STRAD-MO25 complex is transported into the cytosol via exportin proteins (i.e. Exportin 1 and Exportin 7) where it can act on members of the AMP kinase family, regulating metabolism and growth [54].

PKCζ

PKCζ, a serine/threonine kinase, is an atypical isoform from the PKC family of kinases that does not require calcium or diacylglycerol for activation, but is activated following phosphorylation at Thr410 by phosphoinositol-dependent kinase-1/2 (PDK-1/2), a downstream target of PI3 kinase [59]. Following phosphorylation, PKCζ translocates to the nucleus where it increases the phosphorylation of LKB1 at Ser428, resulting in LKB1's translocation to the cytosol [55]. Interestingly, activation of PKCζ, via LKB1, has also been reported to inhibit AKT activity via PTEN phosphorylation in a feedback loop that regulates PI3 kinase signaling [55].

CaMKK2

CaMKK2 (also known as CaMKKβ) is a serine/threonine kinase and a Ca^{2+}/CaM-dependent protein kinase encoded by the CaMKK2 gene. CaMKK2 protein expression is highest in the brain and stimulates signaling cascades involved in neuronal differentiation, migration, memory, and synapse formation [60]. It can influence other physiological processes such as inflammation, glucose homeostasis, and adiposity [61].

CaMKK2 is a 66-68 kDa protein with N- and C-terminal domains, a serine/threonine kinase domain, and a regulatory domain formed by auto-inhibitory and CaM binding regions [61]. At rest, the auto-inhibitory region blocks the active site which is exposed following binding by Ca^{2+}/CaM [62]. Therefore, increase in intracellular calcium levels is a key activator of CaMKK2, resulting in a fully active kinase [61]. Once activated, the most well-known substrates of CaMKK2 are CaMKI

and CaMKIV, but it can also phosphorylate AMPK [61]. Other studies have suggested that CaMKK2 is an alternative upstream AMPK kinase when cells are LKB1 deficient [29, 61].

CaMKK2's Role in PCa

CaMKK2 is consistently reported to be overexpressed in PCa, including CRPC [35, 36] and barely detectable in normal prostate epithelial cells [63, 64]. The AR, which remains a principal regulator of PCa growth, upregulates CaMKK2, promoting glucose uptake, glycolysis and anabolic reactions via AMPK [63]. The link between CaMKK2 and the AR appears to be a feedback loop, whereby CaMKK2 activation maintains AR function, cell growth, and PCa progression (Figure 4) [35]. In addition, CaMKK2 knockdown results in reduced AR activity and PSA expression [35]. Consistent with these observations, a clinical trial with PCa patients found high CaMKK2 expression (in conjunction with low miR-224, a target of the CaMKK2 gene) that resulted in PCa progression and shorter survival times [65]. Overall, the relationship between CaMKK2 and the AR has become an important target for PCa growth and regulation.

CaMKK2 Inhibition

Phosphorylation of CaMKK2 (pCaMKK2) at certain amino acid residues by upstream kinases is thought to prevent the binding of Ca^{2+}/CaM, thus inhibiting catalytic activity.[61, 66-68] These phosphorylation sites include: Ser 100, Ser 495, and Ser 511.[61] Protein kinase A (PKA) and death-associated protein kinase-1 (DAPK1) have been identified as upstream kinases responsible for phosphorylation at one or more of these sites [61].

DAPK

DAPK is a family of serine/threonine kinases that are dependent upon Ca^{2+}/CaM for activation [69]. DAPK1 is considered to be a tumor suppressor protein that increases apoptosis and

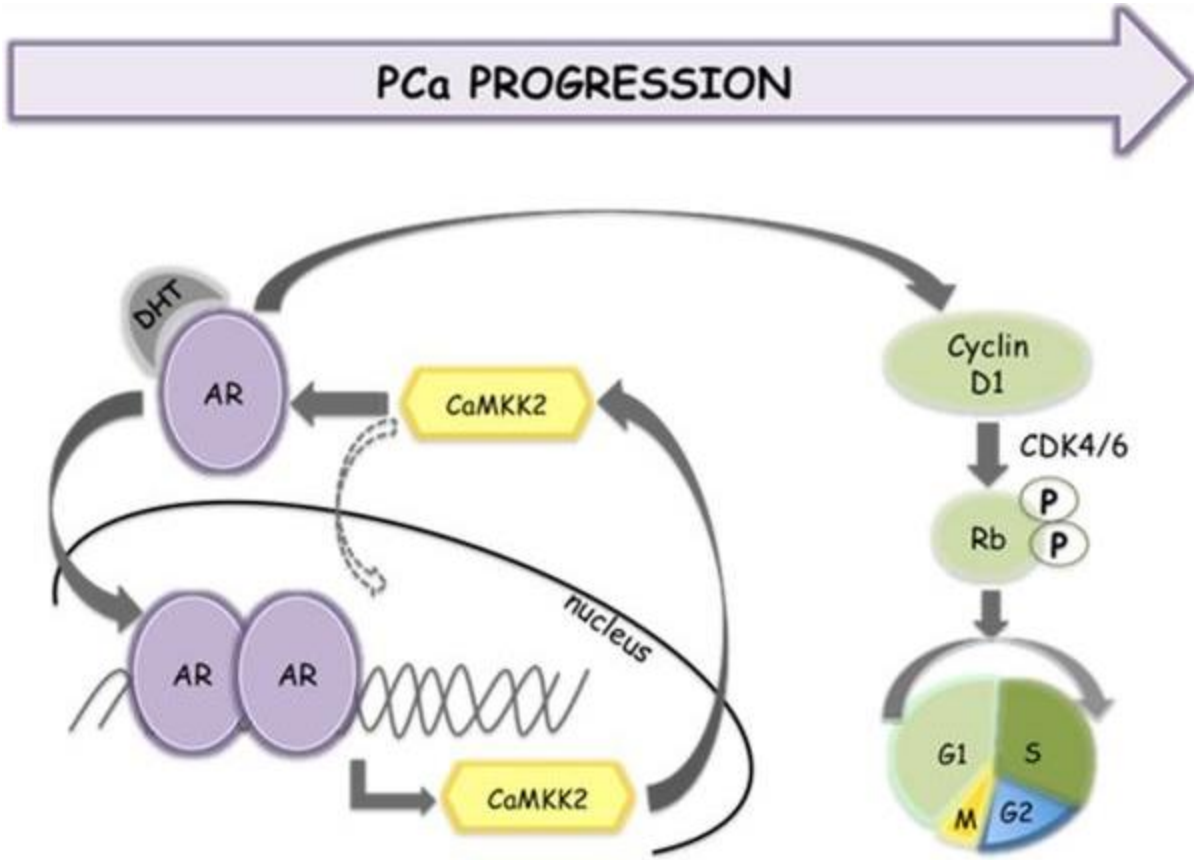

Figure 4. Summary of CaMKK2 and androgen receptor feedback signaling in prostate cancer [35].

autophagy [70]. The rise of intracellular calcium activates a Ca^{2+}/CaM-dependent phosphatase, resulting in the dephosphorylation at Ser 308 of the Ca^{2+}/CaM binding domain of DAPK1, allowing for Ca^{2+}/CaM binding, activating the enzyme [71]. Recently, it was reported that DAPK1 regulates phosphorylation of CaMKK2 at Ser 511, thereby inhibiting catalytic activity and increasing neuronal cell death [66]. However, there is no information in the literature about DAPK-CaMKK2 signaling in cancer.

1.3 Zyflamend

Zyflamend is a polyherbal supplement made from the extracts of ten herbs all of which have been found to have anti-inflammatory and anti-cancer activity. These components include extracts from: ginger (*Zingiber officinale* 12.8%), rosemary (*Rosmarinus offici*nalis 19.2%), oregano (*Origanum vulgare* 5.1%), green tea (*Camellia sinensis* 12.8%), turmeric (*Curcuma longa* 14.1%), barberry (*Berberis vulgaris* 5.1%), Chinese goldthread (*Coptis chinensis* 5.1%), baikal skullcap (*Scutellaria baicalensis* 2.5%), Hu Zhang (*Polygonum cuspidatum* 10.2%), and holy basil (*Ocimum sanctum* 12.8%) (Table 1) [3]. It is a commercial product (New Chapter, Brattleboro, VT) that has not been found to have serious side effects in any preclinical experimental models or effects that were different from placebo in clinical studies.

Zyflamend's Role in Cancer

All herbs within Zyflamend contain constituents that exert anti-inflammatory and anti-cancer activities (i.e. anti-oxidant, anti-angiogenic, anti-proliferative, and apoptotic activities). In human melanoma cells, Zyflamend arrested the cell cycle, inhibited migration, and down-regulated cell growth via apoptosis and autophagy [16]. In a rodent model with pancreatic tumors, Zyflamend, in combination with gemcitabine (a chemotherapeutic drug), significantly reduced tumor volume in comparison with groups that received either Zyflamend or gemcitabine alone, suggesting

Table 1. Components of Zyflamend

Botanical	Representative Bioactive Compounds	Part used	Method of Extraction	Marker/Component Specifications
Rosemary (19.2%) *(Rosmarinus officinalis)*	Rosmarinic acid Ursolic acid Carnosol	Leaf Leaf	Supercritical CO_2 Water and alcohol	Diterpene phenols (22-24%), Essential oils (3-5%) Diterpene phenols (24-26%)
Ginger (12.8%) *(Zingiber officinale)*	Gingerol Shogaol Paradol	Rhizome Rhizome	Supercritical CO_2 Water and alcohol	Pungent compounds (24-35%), Zingiberene (≥8%) Pungent compounds (≥ 3%)
Turmeric (14.1%) *Curcuma longa)*	Curcumoids Turmerones	Rhizome Rhizome	Water and alcohol Supercritical CO_2, water	Curcuminoids (≥11%) Curcumin (≥0.5%), Total essential oil with α- and β-turmerone (70-90%)
Holy Basil (12.8%) *(Ocimum sanctum)*	Ursolic acid	Leaves	Water and alcohol	Ursolic acid (2-3%)
Green Tea (12.8%) *(Camellia sinensis)*	Epigallocatechin Epigallocatechin gallate Epicatechin gallate	Leaves	Water	Polyphenols (≥45%)
Hu Zhang (10.2%) *(Polygonum cuspidatum)*	Resveratrol	Radix/Rhizome	Water and alcohol	Resveratrol (≥8%)
Chinese Goldthread (5.1%) *(Coptis chinensis)*	Berberine Coptisine	Root	Water and alcohol	Berberine (≥6%)
Barberry (5.1%) *(Berberis vulgaris)*	Berberine	Root bark	Water and alcohol	Berberine (≥6%)
Oregano (5.1%) *(Origanum vulgare)*	Carvacrol Linalool Rosmarinic acid Thymol	Leaf	Supercritical CO_2, water	Essential oils (8-12%), Phenolic antioxidants (≥4.5%)
Baikal Skullcap (2.5%) *(Scutellaria baicalensis)*	Baicalin Baicalein Wogonin	Root	Water and alcohol	Baicalin (≥17%), Baicalein (≥1.5%), Wogonin (≥0.4%)

As previously reviewed by Whelan *et al* (2017) [3].

Zyflamend may sensitize cancer cells to chemotherapeutic drugs [14]. A major target in cancer treatment is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor that regulates multiple tumorigenic genes (i.e. anti-apoptotic genes, cell cycle genes, angiogenesis, and inflammatory cytokines) [13]. Zyflamend was found to down-regulate NF-κB and suppressed cell growth in leukemia, lung adenocarcinoma cells, and pancreatic tumors [13, 14]. In addition, Zyflamend protected pancreatic β-cells from immune cell destruction in a rodent model pre-disposed to Type 1 diabetes by a proposed process that inhibited NF-κB activation [72].

Zyflamend's Role in PCa

Although Zyflamend exerts anti-cancer activity in multiple cancers, particular interest has developed in its role against PCa. The first study published with Zyflamend involved a case report in a 70-year-old African American male with high grade prostatic intraepithelial neoplasia (HGPIN), a strong precursor of PCa. After 18 months of Zyflamend supplementation, the patient was PIN free and had decreased levels of cyclooxygenase (COX-2) activity, an important enzyme shown to be over-expressed in PCa [18]. This led to a phase 1 clinical trial which examined the safety and tolerability of Zyflamend supplementation for 18 months in men with HGPIN. By the end of the study, 48% of subjects had a 25-50% reduction in PSA levels, 60% had benign tissue versus 26.7% with HGPIN, while 13.3% developed PCa. No serious side effects were reported, suggesting daily Zyflamend supplementation may be a safe treatment for HGPIN [20]. More recently, a clinical trial of four patients with advanced PCa who no longer responded to standard treatments (i.e., HDT, chemotherapy, surgery, immunotherapy) were treated orally with metformin and/or Zyflamend. By the end of the study, all patients taking Zyflamend, metformin, or the combination had dramatic decreases in circulating PSA levels [9].

The positive responses of Zyflamend's role against PCa has led to further *in vivo* and *in vitro* experimentation to determine its mechanisms of actions, particularly in CRPC. Zyflamend inhibits CRPC growth by regulating critical mediators of inflammation, energetics, cell survival, and other signaling pathways, as summarized in Figure 5 [3]. Perhaps the most critical mediator of human PCa is AR activation in both androgen dependent and CRPC tumors. In a model of CRPC, human equivalent doses of Zyflamend down-regulates the androgen-receptor and its nuclear localization [6]. In addition, combined treatment of Zyflamend and bicalutimide (an anti-AR drug), synergistically inhibited PCa cell growth [21].

Other attractive targets in cancer treatment is to arrest the cell cycle by increasing expression of CDK inhibitors such as p21 and p27. Zyflamend increased p21 and p27 expression, resulting in reduced cell proliferation [8]. These effects may be due to its ability to inhibit histone deacetylases (HDAC) [8]. The hyper-acetylation of histones relaxes the chromatin around DNA, making it more accessible for transcription factors to increase the expression of CDK inhibitors, like p21. These results are important since HDACs are reported to be expressed higher in CRPC than in androgen-dependent PCa models [3].

Inflammation has long been associated with many cancers, including PCa [73]. Zyflamend down-regulates multiple targets of pro-inflammatory mediators including NF-κB, STAT3, and enzyme activities of cyclooxygenase-1 and 2 (COX-1/2) and lipoxygenases (LOX) such as 5-LOX and 12-LOX [17, 19, 20]. These results have been recapitulated in multiple laboratories studying Zyflamend's anti-inflammatory role in models other than PCa, such as pancreatic cancer, diabetes, obesity, and oral cancer [12, 14, 72, 74].

Zyflamend's multiple anti-cancer mechanisms could be due to the combination of extracts, rather

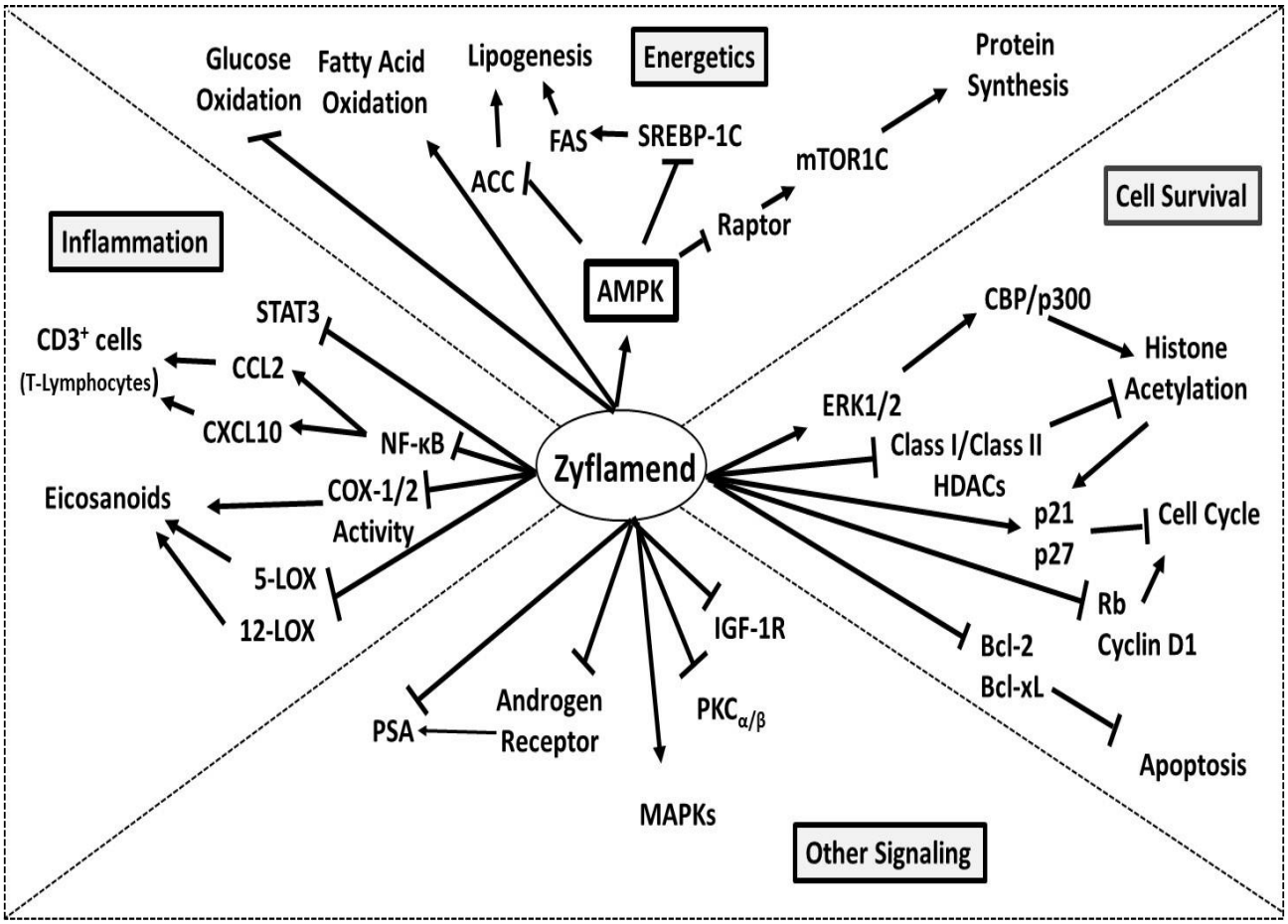

Figure 5. Summary of Zyflamend's mechanisms of actions in prostate cancer [3].

than isolated bioactives derived from those extracts. Components of Zyflamend exerted synergistic activity at physiologically nanomolar concentrations in comparison with individual bioactives at equivalent physiological concentrations [22]. For example, turmeric and Chinese Goldthread (two components of Zyflamend) inhibited CRPC proliferation and NF-κB signaling greater than their major bioactives in isolation (curcumin and berberine, respectively) [22]. These results suggest that other compounds of turmeric and Chinese Goldthread are responsible for this increased sensitivity [3, 22]. However, confirming which combinations of extracts in Zyflamend accounts for most of its biological activity is not feasible. For example, there are 1024 possible combinations of the 10 extracts in Zyflamend (as summarized in Table 2). Even testing only 2 items would result in 57 possible combinations. Since the research in this thesis took ~2 years using only two combinations (control and all 10 extracts), it would be impractical to explore multiple combinations. Important to this research is the fact that the components in Zyflamend act synergistically, at physiologically relevant concentrations, against multiple cancers, including CRPC.

Irregular cellular energetics is a hallmark of cancer that promotes rapid tumor cell proliferation. Zyflamend is an AMPK activator that switches the energetics of cells from an anabolic to a more catabolic state, thereby inhibiting cell viability in CRPC [10]. AMPK activation by Zyflamend inhibited critical mediators of lipogenesis including acetyl CoA carboxylase (ACC), sterol regulatory element binding protein-1c (SREBP-1c), and fatty acid synthase (FAS) (Figure 6) [10]. These results are important since PCa relies on increased fatty acid synthesis and lipogenesis for building new cell membranes [44, 45]. In addition, AMPK down-regulated mTORC1 signaling, an important pathway in protein synthesis via phosphorylation of its regulatory subunit, raptor [10]. Overall, Zyflamend-stimulated AMPK, depriving CRPC cells from synthesizing macronutrients necessary for rapid cell proliferation.

Table 2. The number of possible combinations using 1-10 extracts in Zyflamend.

Possible Combinations of Herbal Extracts	Calculation
For all possible combinations using all 10 (includes negative (vehicle) and positive (all 10) controls:	$2^{10} = 1024$
For all possible combinations using any 9 out of 10 plus negative (vehicle) and positive (all 10) controls:	1022+1+1=1024
For all possible combinations using any 8 out of 10 plus negative (vehicle) and positive (all 10) controls:	1012+1+1=1014
For all possible combinations using any 7 out of 10 plus negative (vehicle) and positive (all 10) controls:	967+1+1=969
For all possible combinations using any 6 out of 10 plus negative (vehicle) and positive (all 10) controls:	847+1+1=849
For all possible combinations using any 5 out of 10 plus negative (vehicle) and positive (all 10) controls:	637+1+1=639
For all possible combinations using any 4 out of 10 plus negative (vehicle) and positive (all 10) controls:	385+1+1=389
For all possible combinations using any 3 out of 10 plus negative (vehicle) and positive (all 10) controls:	175+1+1=177
For all possible combinations using any 2 out of 10 plus negative (vehicle) and positive (all 10) controls:	55+1+1=57
For all possible combinations using any 1 out of 10 plus negative (vehicle) and positive (all 10) controls:	10+1+1=12

Note: These calculations are derived from the following basic formula: $\frac{(k!)}{([k-r]!)\,(r!)}$

k = the number of herbal extracts (10)
r = the number of extracts to be used out of the 10
! = factorial of the number (i.e., "3!" is equivalent to 3 x 2 x 1)

For example: to determine the possible combinations of 5 items (n=5 with each combination) of the 10 extracts, the formula would be:

$\frac{(10!)}{([10-5]!)\,(5!)}$ or $\frac{(10 \cdot 9 \cdot 8 \cdot 7 \cdot 6 \cdot 5 \cdot 4 \cdot 3 \cdot 2 \cdot 1)}{(5 \cdot 4 \cdot 3 \cdot 2 \cdot 1)\,(5 \cdot 4 \cdot 3 \cdot 2 \cdot 1)} = 252$

However, to calculate all possible combinations of 5 *or less* (since the calculation above is only for n=5), you have to do the same calculation for n=4, n=3, n=2 and n=1, and add these all up and then include negative (vehicle) and positive (all 10) controls for the final calculation.
When only calculating all possible combinations for all 10 items (including controls), the following formula can be used: 2^{10}.

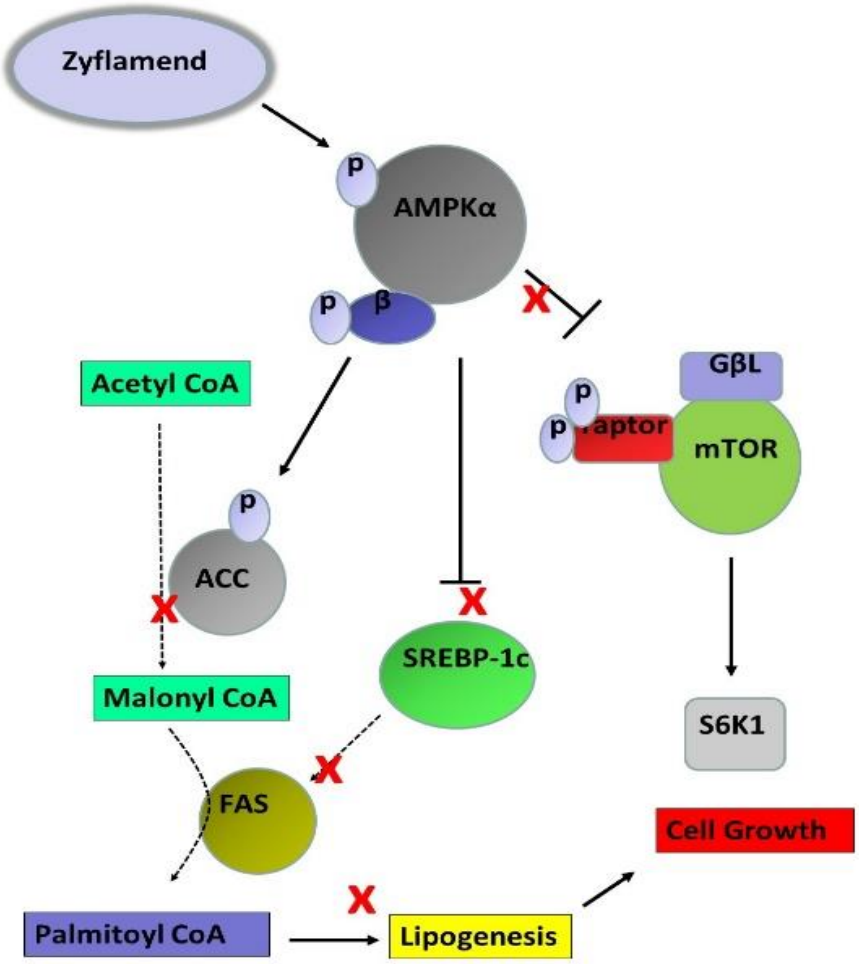

Figure 6. Zyflamend activates AMPK and downstream signaling events. AMPK activation by Zyflamend inhibits lipogenesis by phosphorylating ACC and down-regulating SREBP-1c, a transcription factor needed for the expression of lipogenic enzymes such as fatty acid synthase (FAS). In addition, AMPK inhibits protein synthesis by down-regulating mTORC1 via phosphorylation of its regulatory subunit, Raptor. Overall, AMPK activation by Zyflamend results in a metabolic switch that deprives tumor cells of macromolecules necessary for proliferation [10].

1.4 Research Objectives

Specific Aims

Prostate cancer is the third most deadly cancer of men in the United States. Current standard treatments of men with metastatic, castrate-resistant forms of PCa are unpromising, particularly following metastasis, with a survival rate of <2 years. Zyflamend, is a poly-herbal compound that has been shown to be safe and effective in inhibiting CRPC growth *in vitro* and in preclinical experimental models. More specifically, Zyflamend's tumor suppressive effects are, in part, due to the phosphorylation and activation of AMPK, a tumor suppressor protein that inhibits lipogenesis and protein synthesis for rapidly dividing and proliferating PCa cells. The primary objective of this study is to interrogate how Zyflamend regulates AMPK phosphorylation in a model of CRPC.

Currently, two kinases have been identified as being important in PCa that phosphorylate AMPK at Thr172 of the catalytic α subunit: CaMKK2 and LKB1. CaMKK2 is involved in tumor promotion and regulates AMPK phosphorylation by a rise of intracellular calcium levels, while LKB1 regulates AMPK phosphorylation upon energetic stress.

The central hypothesis, based on preliminary data from our laboratory, is that Zyflamend does not activate AMPK via CaMKK2, but instead increases its activation via LKB1. Thus, the overall objective was to interrogate how Zyflamend activates AMPK in a model of CRPC and determine the roles of LKB1 and CaMKK2 in this activation.

Specific Aim 1: To determine if the phosphorylation and activation of AMPK by Zyflamend is mediated, in part, by CaMKK2 using pharmacologic and/or molecular techniques in the presence and absence of Zyflamend.

Specific Aim 2: To determine if the phosphorylation and activation of AMPK by Zyflamend is mediated, in part, by LKB1 using pharmacologic and/or molecular techniques in the presence and absence of Zyflamend.

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Chapter II

Concurrent regulation of LKB1 and CaMKK2 in the activation of AMPK by Zyflamend, a polyherbal mixture with anticancer properties.

Abstract

Zyflamend, a select blend of 10 herbal extracts, effectively inhibits tumor growth using preclinical models of castrate-resistant prostate cancer (CR-PCa), mediated in part by activating AMPK, a master energy sensor of the cell. Currently, two predominant upstream kinases are known to phosphorylate/activate AMPK at Thr172: LKB1, a known tumor suppressor, and CaMKK2, a tumor promotor over-expressed in a number of cancers. Thus, the overall objective was to interrogate how Zyflamend activates AMPK and determine the roles of LKB1 and CaMKK2 in this activation, by targeting upstream mediators of both kinases ± Zyflamend using pharmacologic and molecular techniques in CWR22Rv1 (CR-PCa cell line) and HeLa (LKB1-null cell line) cells. Zyflamend-mediated activation of AMPK appears to be LKB1 dependent, while coordinately and negatively regulating CaMKK2 activity. Zyflamend failed to rescue the activation of AMPK in the presence of chemical and molecular inhibitors of LKB1 (radicicol & siLKB1), an effect not observed in the presence of inhibitors of CaMKK2 (STO-609, EGTA) in CWR22Rv1 cells. Using LKB1-null and catalytically dead LKB1-transfected HeLa cells that constitutively express CaMKK2, ionomycin (activator of CaMKK2) increased phosphorylation of AMPK, but Zyflamend had no effect. Zyflamend appears to inhibit CaMKK2 by DAPK-mediated phosphorylation at Ser511, an effect prevented by a DAPK inhibitor. Alternatively, Zyflamend increased pAMPK only in HeLa cells transfected with wild type LKB1. Zyflamend increased phosphorylation of PKCζ, a known activator of LKB1, and inhibition of PKCζ reduced LKB1 phosphorylation. Using various constructs of HeLa cells, nuclear and cytosolic localization of LKB1 and PKCζ, respectively, were reversed following Zyflamend treatment, consistent with phosphorylation of AMPK by LKB1 in the cytosol. These results suggest that Zyflamend's activation of AMPK is mediated by LKB1, possibly via activation of PKCζ, but independent of CaMKK2. Moreover, Zyflamend inhibits CaMKK2 activity by a mechanism involving DAPK phosphorylation of CaMKK2 at Ser511.

2.0 Introduction

Prostate cancer is the third leading cause of death for men in the United States [1]. While early stages of the disease are treatable, with 5-year survival rates near 100%, prognosis for advanced forms are less promising [2]. Initially, prostate cancer cells rely on androgens for growth, and chemically-mediated deprivation (hormone deprivation therapy) is a common therapy that results in cancer regression [3]. Relapse in the absence of androgens (castrate-resistant prostate cancer) is inevitable for most individuals and is associated with increased expression and activation of the androgen receptor, a major determinant in survival [4, 5]. Due to the poor prognosis of castrate-resistant prostate cancer, concomitant use of natural products to enhance effectiveness is being explored clinically and experimentally [4, 6-10].

Zyflamend (New Chapter, Inc. Brattleboro, VT) is a poly-herbal supplement derived from the extracts of ten different herbs: rosemary, turmeric, holy basil, ginger, green tea, hu zhang, barberry, oregano, Chinese goldthread, and baikal skullcap. Most research using Zyflamend has focused its effects on a variety of cancer models, including oral [11], mammary [12], bone [13], pancreas [14, 15], skin [11, 16], colorectal [15], with an emphasis on prostate [6-9, 17-21], and its beneficial effects appear to be related to the synergy of action of its components [22]. The effects of Zyflamend and its mechanisms on prostate cancer has been reviewed elsewhere and can be summarized in Figure 1 [3]. Zyflamend inhibits signaling pathways of inflammation, affects cell survival by enhancing apoptotic and tumor suppressor genes, epigenetically modifies histones, down regulates the androgen receptor and influences the energetics of the cell. The latter pathways are critically important in cancer as rapidly dividing cells rely on the increased synthesis of macromolecules (lipids, proteins, nucleotides, etc) (as reviewed in [23]).

5'-adenosine monophosphate-activated protein kinase (AMPK) is a key regulator of energy in the cell and responds to deficits in adenosine triphosphate (ATP). The protein contains a catalytic subunit (α-subunit), and two regulatory subunits, β and γ-subunits. Under conditions of energy stress the following occurs, *(i)* increased levels of AMP or ADP bind to the γ-subunit causing allosteric activation of the protein (ATP is a competitive inhibitor), *(ii)* increased affinity for upstream kinases that target phosphorylation at Thr172 of the α-subunit (increasing catalytic activity >100 fold), and *(iii)* reduced affinity for phosphatases that are involved in dephosphorylation at Thr172 [24-26]. When activated, AMPK is instrumental in inhibiting anabolic pathways that consume ATP, such as lipogenesis and protein synthesis, and enhances catabolic pathways that generate ATP, such as fatty acid oxidation [23, 27].

Recently, it was determined that tumor suppressor properties of Zyflamend is associated with the activation of AMPK and its downstream signaling [10]. This involves inhibiting the mammalian target of rapamycin complex-1 (mTORC1), the expression of fatty acid synthase and its regulatory transcription factor (SREBP1c), and inhibiting the activity of acetyl CoA carboxylase (ACC), a key regulator of the lipogenic pathway. What is not known is how Zyflamend upregulates AMPK. Four kinases have been identified that activate AMPK at Thr172, liver kinases B1 (LKB1), calcium-calmodulin kinases kinase-2 (CaMKK2), transforming growth factor-β activated protein kinase-1 (TAK1) and mixed lineage kinase 3 (MLK3) [28-32]. LKB1 and CaMKK2 are important in a number of cancers, including castrate-resistant prostate cancer (as reviewed in [33]), while the involvement of TAK1 and MLK3 has yet to be determined. LKB1 responds to increases in AMP and ADP, while increases in intracellular calcium is needed for activation of CaMKK2 without requiring elevation in AMP or ADP.

Interestingly, while both LKB1 and CaMKK2 are involved in activating AMPK, their effects on cancer appear to be quite different. LKB1 has anticancer properties because its mutation/deletion is associated with a variety of cancers [34]. CaMKK2, on the other hand, is overexpressed in a number of cancers, including castrate-resistant prostate cancer [35-37]. Therefore, the overall objective of this paper is to interrogate how Zyflamend activates AMPK in a model of castrate-resistant prostate cancer and the roles LKB1 and CaMKK2 play in that activation.

2.1 Materials and Methods

Materials

Zyflamend (New Chapter, Inc. Brattleboro, VT) is composed of the extracts the following herbs (w/w): rosemary (*Rosmarinus offici*nalis 19.2%), turmeric (*Curcuma longa* 14.1%), holy basil (*Ocimum sanctum* 12.8%), ginger (*Zingiber officinale* 12.8%), green tea (*Camellia sinensis* 12.8%), hu zhang (*Polygonum cuspidatum* 10.2%), barberry (*Berberis vulgaris* 5.1%), oregano (*Origanum vulgare* 5.1%), Chinese goldthread (*Coptis chinensis* 5.1%), and baikal skullcap (*Scutellaria baicalensis* 2.5%). Detailed description and characterization of the preparation of Zyflamend and quality assurance of the mixture has been described previously in detail [7]. Dulbecco's Modified Eagle Medium (DMEM), G418, penicillin/streptomycin, puromycin, fetal bovine serum (FBS) and trypsin were purchased from Invitrogen (Carlsbad, CA). Cloning vectors were purchased from Addgene (Cambridge, MA) Antibodies for PKC-zeta (PKC-ζ), LKB1, phospho-LKB1, GFP, Histone B, Flag, and Tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). AMPK and phospho-AMPK were from Cell Signaling Technology (Beverly, MA). The following chemical reagents were purchased: 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (AdipoGen Life Sciences, San Diego, CA); 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) and

ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (Thermo Scientific, Rockville, IL); STO-609, radicicol, and PKC-ζ Pseudo-substrate Inhibitor (Santa Cruz Biotechnology, Dallas, TX); ionomycin (Sigma-Aldrich, St. Louis, MO); and Death Associated Protein Kinase Inhibitor (DAPKi) (Merck Millipore, Billerica, MA).

Cell Culture

CWR22Rv1 cells (American Type Culture Collection, Rockville, MD), a human-derived castrate-resistant PCa cell line, were cultured in RPMI 1640 media, supplemented with 10% FBS. To mimic an androgen-depleted state, the cells were incubated overnight with 0.5% FBS. HeLa cells (ATCC, Rockville, MD), a human-derived cervical cancer cell line that do not express LKB1, and HCT 116 cells (ATCC, Rockville, MD), a human derived colorectal cancer cell line, were cultured in DMEM media supplemented with 10% FBS and 25 mM glucose. For experimental conditions with HeLa cells and HCT 116 cells, All cells were incubated under an atmosphere of 5% CO_2, at 37°C. For activation of AMPK via LKB1-dependent or CaMKK2-dependent pathways, cells were treated with AICAR (a cell permeable analog of AMP) (1 mM, 1 hr) or ionomycin (calcium ionophore) (1 µM, 1 hr), respectively. For experiments using inhibitors of CaMKK2, cells were pre-treated with the selective CaMKK2 inhibitor STO-609 (10 µM, 30 min) or the calcium chelators BAPTA-AM (30 µM, 30 min) or EGTA (2 mM, 30 min). For inhibition of LKB1 or death-associated protein kinase (DAPK), cells were pre-treated with radicicol (5 µM, 24 hr) or DAPKi (20 µM, 24 hr), respectively. For inhibition of PKC-ζ, cells were pre-treated with the selective PKC-ζ pseudo-substrate inhibitor (5 µM, 30 min). For all experiments involving Zyflamend, cells were treated with Zyflamend at 200 µg/mL for 30 min unless otherwise indicated.

Down regulation of LKB1 by small interfering RNA

CWR22Rv1 cells were seeded in RPMI medium containing 10% FBS and incubated overnight before media was replaced with RNA transfection medium containing 0.5% FBS. Cells were transfected with 20 nmol of siRNA targeting LKB1 (Thermo Scientific/Dharmacon #L-005035-00) and a siRNA non-targeting control (Thermo Scientific/Dharmacon #D-001810-10-05). Western blot analysis confirmed the efficiency of knockdown to be 76% 48 hr after transfection, at which time cells were treated with a vehicle or Zyflamend (200 ug/mL) for 30 min.

Overexpression of LKB1 in HeLa cells

When indicated human WT or catalytically dead (KD) mutants of LKB1 were transfected into HeLa cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) following manufacturer's guidelines. Cells were cultured for additional 48 hr prior experiments. For total protein lysates, cells were lysed in radio-immunoprecipitation assay buffer (RIPA: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate and protease inhibitors). Lysates were clarified by centrifugation at 13,000 rpm for 10 min, and protein concentrations were determined using a bicinchoninic acid assay kit (Pierce Chemical). Proteins (10 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8%-12%) [75], transferred to polyvinylidene difluoride (PVDF) membranes and immunodetected using the indicated antibodies. Proteins were detected using enhanced chemiluminescence (Amersham Biosciences). Resulting immunoreactive bands were quantified using FluorChem Q Imaging software (Alpha Innotech).

Subcellular fractionation

Following Zyflamend treatment, fractionation was performed in HeLa cells as described previously with modifications [76, 77]. Briefly, cells were washed with cold buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and resuspended in lysis buffer B (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, 0.1 DTT, 5% freshly added percoll, 0.01% digitonin, 1 mM PMSF and cocktail of protease inhibitors, pH 7,4). Membranes were broken using a dounce homogenizer (200 strokes/sample). Debris and unbroken cells were removed by centrifugation (500g for 10 min) and supernatants were then centrifuged (2 500g, 5 min) to separate nuclei (pellet). Supernatants were centrifuged again (15 000g, 15 min) to separate mitochondria. Nuclear fraction was resuspended in RIPA buffer containing proteases inhibitors. Cellular distribution and translocation of the indicated proteins were analyzed by SDS-PAGE and Western blot as described above. Purity of nuclear and cytoplasmic fractions was verified using antibodies against histone B and tubulin, respectively.

Western blotting

Cells were lysed in RIPA lysis buffer (Thermo Scientific, Rockford, IL). Protein concentration was measured using a Bradford protein assay (Thermo Scientific, Rockford, IL). Equal amount of protein (30 µg) were separated by 8% SDS-PAGE and transferred to a PVDF membrane by electroblotting. Membranes were blocked by 5% non-fat dry milk (LabScientific, Highlands, NJ) in 0.1% Tris-buffered saline-Tween-20 (TBST) for 1 hr at room temperature and incubated in TBST containing primary antibodies overnight at 4°C. Membranes were incubated with anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase (HRP) (Cell Signaling Technology, Danver, MA) for 1 hr at room temperature. Protein expression was detected with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and membranes were exposed and analyzed via *Li-Cor Odyssey FC* imaging

system (Li-Cor, Lincoln, NE). Antibodies against p-AMPKα (Thr172), AMPKα, pACC (Ser79), ACC, pLKB1 (Ser428), LKB1, pPKCς (Thr410), PKCς, and pCaMKKβ (Ser511) were used to detect target protein level at 1:1000. β-Actin or GAPDH (Santa Cruz Biotechnology, Dallas, TX) was used as the loading control.

ATP Assay

Cellular ATP concentration was determined using a fluorometric ATP assay kit (BioRad, Milpitas, CA) following the manufacturer's instructions, and fluorescence was read at 525 nm on a Glowmax Multi Detection System (Promega Corporation, Madison, WI).

Statistics

For Western blot, protein was analyzed from 3 independent samples and presented as mean±SEM. For ATP concentration, results are presented as mean±SEM. For multiple comparisons, data was analyzed using IBM SPSS Statistics 24 and tested by One-Way ANOVA followed by a Tukey's post-hoc test. Two-group comparisons were analyzed by two-tailed Student's T-test. Results were considered statistically significant at $p<0.05$.

2.2 Results

Effect of Zyflamend on cell proliferation, ATP levels and AMPK phosphorylation in CWR22Rv1 cells.

Zyflamend (200 μg/mL) inhibited cell proliferation in CWR22Rv1 cells in a concentration and time dependent manner (data not shown) [8]. Similar results were observed in a variety of immortalized prostate-derived cells lines [8] and in the HCT116 colorectal cell line (Fig. S.1A). Because Zyflamend has been shown to change the energetics of CWR22Rv1 cells [3, 10], levels of ATP were determined. In the presence of Zyflamend, ATP levels were reduced by

~40% (Fig. 7A) and AMPK phosphorylation (Thr172) increased ~8 fold within 30 min (Fig. 7B and C), results replicated in HCT116 cells (Fig. S.1B and C).

AMPK activation by CaMKK2 in the presence and absence of Zyflamend in CWR22Rv1 cells.

Zyflamend significantly increased the phosphorylation of AMPK (Thr172) (Fig. 8A and B) and its downstream target ACC (Ser79) (Fig. 8C and D), results unaffected by pretreatment with the CaMKK2 inhibitor STO-609 (Fig. 8A-D, lane 4/bar 4). To confirm that the activation of AMPK is independent of CaMKK2, cells were pre-treated with the calcium chelator BAPTA-AM, as CaMKK2 activation is dependent upon intracellular calcium. Pretreatment with BAPTA-AM failed to prevent phosphorylation of AMPK in the presence of Zyflamend (Fig. 8E and F). (Similar results were obtained using EGTA, another calcium chelator (Fig. S.2). Zyflamend increased the phosphorylation of CaMKK2 at Ser511 (Fig. 8G), a phosphorylation site that results in its inhibition and reported to be mediated, in part, by DAPK [66]. Pretreatment with a DAPK inhibitor attenuated Zyflamend's ability to increase phosphorylation of CaMKK2 at Ser511 in a time-dependent manner (Fig. 8H-J).

AMPK activation by LKB1 in the presence and absence of Zyflamend in CWR22Rv1 cells.

Zyflamend significantly increased the phosphorylation of LKB1 (Ser428) (Fig. 9A), AMPK (Thr172) and ACC (Ser79) (Fig. 9B-F). In the presence of radicicol, a non-specific inhibitor of LKB1, phosphorylation of AMPK failed to be fully restored following treatment with Zyflamend (Fig. 9B and C). Likewise, knockdown of LKB1 by siRNA inhibited Zyflamend-mediated phosphorylation of AMPK and its downstream target ACC (Fig. 9D-F).

Zyflamend-induced AMPK phosphorylation is LKB1 dependent.

To confirm that Zyflamend-mediated phosphorylation of AMPK is LKB1 dependent and CaMKK2 independent, we treated LKB1-null HeLa cells, that constitutively express CaMKK2, with AICAR (an activator of AMPK that is commonly used as a positive control), ionomycin (activator of CaMKK2) and Zyflamend (Fig. 10). This experiment was repeated in HeLa cells that were stably transfected with wild-type (WT) LKB1 or a protein that was catalytically dead (KD) (mutants of the human LKB1), using two different constructs, Flag-tagged or green fluorescent protein (KD-LKB1 Flag or KD-LKB1 GFP, respectively) (Fig. 10B). In both mutants, lysine 78 was mutated to isoleucine, abolishing auto-phosphorylation and activation of LKB1 [78, 79]. Ionomycin, but not AICAR or Zyflamend, induced phosphorylation of AMPK in LKB1-null HeLa cells (control) (Fig. 10B, columns 1-5). Following transfection with WT-LKB1 (Fig. 10B, columns 6-8), Zyflamend induced phosphorylation of LKB1 and AMPK, an effect more pronounced with co-treatment of AICAR. However, no phosphorylation of LKB1 and AMPK was observed in the KD mutants following treatment with Zyflamend and Zyflamend+AICAR (Fig. 10B, columns 9-14).

Zyflamend-mediated LKB1 phosphorylation is linked to PKCzeta.

In an effort to determine how Zyflamend may be mediating the phosphorylation of LKB1, PKCς was investigated as a possible upstream target (Fig. 11). Phosphorylation of PKCς and LKB1 increased when CWR22Rv1 cells were treated with Zyflamend (Fig. 11A). However, in the presence of a highly selective PKCζ pseudo-substrate inhibitor, phosphorylation of LKB1 could not be restored to control levels following Zyflamend treatment (Fig. 11B and C). Using our informative HeLa cell constructs, null for LKB1 and cells transfected with WT-LKB1 and KD-LKB1, we further investigated the relationship between PKCς and LKB1 (Fig. 11D). In HeLa cells devoid of LKB1 (control cells), PKCς is located in the cytosol (Fig. 11D, row 1, column 3), but appears to translocate to the nucleus upon treatment with Zyflamend (Fig. 11D, row 1,

column 2). In cells transfected with WT-LKB1, PKCζ is located in the cytosol (Fig. 11D, row 1, column 7) and LKB1 is located in the nucleus (Fig. 11D, row 2, column 5). Following Zyflamend treatment, their locations switch, where PKCζ translocates to the nucleus (Fig. 11D, row 1, column 6) and LKB1 is found in the cytosol (Fig. 11D, row 2, column 8). This translocation following Zyflamend treatment appears to be independent of a catalytically active protein, as the same results were observed with the KD-LKB1 mutant (Fig. 11D, columns 9-12).

2.3 Discussion

Zyflamend is a unique blend of ten herbal extracts with tumor suppressor properties whose biochemical and physiological effects have been replicated in different laboratories, at different times, using different lots, with similar doses/concentrations [6-8, 10, 11, 13-17, 19, 21, 74] . The quality control of this preparation has been summarized elsewhere [7] and is most likely responsible for the reproducibility of results. While there is clinical evidence for the beneficial effects of Zyflamend on prostate cancer [9, 18, 20], it is not possible to tease out the contributions of each constituent as their effects can be dramatically enhanced when used in combination [22].

Castrate-resistant prostate cancer is the focus of this research and the research in our laboratory. To study mechanisms of action, we use human prostate cancer cells derived from the CWR22 lineage [6-8, 10, 80, 81]. Similar to the progression of human prostate cancer, these cells are originally androgen dependent and can transform to a castrate-resistant line *in vivo* (i.e., CRW22R) following hormone ablation [81-83]. Unlike other prostate cancer cell lines, the CWR22Rv1 cells express a constitutively active androgen receptor and prostate specific antigen (PSA), characteristics shared by castrate-resistant prostate cancer in humans.

The effectiveness of Zyflamend on prostate cancer rests, in part, with its ability to upregulate AMPK, an effect observed in other cell types [10]. In addition, case studies from M.D. Anderson Cancer Center report dramatic reductions in PSA levels following Zyflamend and/or metformin treatment in patients whose prostate cancer no longer responds to standard therapies [9]. Metformin is a known activator of AMPK [41]. However, the mechanism as to how Zyflamend upregulates AMPK is unknown, although many of its constituents have been shown to independently activate AMPK by modifying mitochondrial ATP production (as reviewed in [41]). This is the first paper to delineate the coordination of potential upstream pathways involved in the activation of AMPK, *viz.,* LKB1 [27] and CaMKK2 [29], especially by natural products.
The role of AMPK in prostate cancer is controversial in the sense that upstream kinases responsible for its activation appear to have contradictory effects on cancer [33]. CaMKK2 is a known tumor promotor whose expression is linked to the upregulation of the androgen receptor, a key step in castrate-resistant prostate cancer [4, 35, 36]. Interestingly, Zyflamend down regulates the androgen receptor and its nuclear localization [6]. While CaMKK2 has a number of phosphorylation sites, phosphorylation at Ser511, a sight adjacent to the Ca^{+2}-calmodulin regulatory domain, prevents autophosphorylation, inhibiting catalytic activity [66]. DAPK is a kinase in the serine/threonine family of kinases involved in apoptosis and is known to suppress tumor growth and metastasis [84]. A downstream target of DAPK is CaMKK2 with its phosphorylation site at Ser511 [66, 68].

In contrast, LKB1 exhibits tumor suppressor properties, where loss of LKB1 is involved in a variety of cancers [25, 34]. LKB1-mediated activation of AMPK is dependent upon increases in the AMP(ADP):ATP ratios, an effect observed with Zyflamend. LKB1 contains a nuclear localization domain and is typically (but not exclusively) found in the nucleus. Following activation, LKB1 co-localizes with STE20-related adaptor (STRAD) protein and scaffolding mouse 25 (MO25) protein and translocates to the cytosol where it exerts its kinase activity on a

number of downstream targets, including AMPK [49, 54]. Nuclear export, in part, appears to involve phosphorylation at Ser428 by PKCζ [55].

A key finding from this research is that Zyflamend antithetically regulates two parallel pathways important in the phosphorylation of AMPK that is potentially important in castrate-resistant prostate cancer. These effects are summarized in Figure 6. Zyflamend-mediated activation of AMPK appears to be LKB1 dependent, while coordinately and negatively regulating CaMKK2 activity. This was observed using LKB1-null and KD-LKB1 transfected HeLa cells that constitutively express CaMKK2. The addition of ionomycin (activator of CaMKK2) robustly increased phosphorylation of AMPK, but Zyflamend (with and without AICAR, an AMP analog) had no effect. Our results suggest that Zyflamend inhibits CaMKK2 following DAPK-mediated phosphorylation at Ser511, as this effect is prevented by the presence of a DAPK inhibitor.

On the other hand, Zyflamend robustly increased the phosphorylation of AMPK only in HeLa cells transfected with WT LKB1. Using the various constructs of the HeLa cells, we confirmed nuclear localization of LKB1, with translocation to the cytosol following Zyflamend treatment (Fig. 11). Zyflamend increased phosphorylation of PKCζ, a known activator of LKB1, and inhibition of PKCζ reduced LKB1 phosphorylation in the presence of Zyflamend.

Importantly, translocation of PKCζ from the cytosol to the nucleus occurred concomitantly. These results help explain why inhibitors of CaMKK2 (STO-609, EGTA) failed to prevent the activation of AMPK in the presence of Zyflamend in CWR22Rv1 cells. This was due to the simultaneous activation of LKB1, and this was confirmed when activation of AMPK (in the presence of Zyflamend) was not completely rescue following inhibition (radicicol) and knockdown (siRNA) of LKB1.

In summary, Zyflamend has been shown to inhibit castrate-resistant prostate cancer, in part, through the activation of AMPK [10]. In conjunction with reducing ATP levels, this activation is mediated by the tumor suppressor protein LKB1, via activation of PKCζ. Simultaneously, Zyflamend inhibits CaMKK2, a tumor promotor that is over-expressed in many cancers, including castrate-resistant prostate cancer. This inhibition appears to be mediated by DAPK.

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Summary

In summary, PCa is the third leading cause of cancer-related deaths in the United States. The effectiveness of standard treatments, particularly for metastatic PCa at the castrate-resistant stage, are unpromising with a survival rate of less than 2 years. However, a growing interest is the use of natural products that can augment standard therapies of CRPC.

Zyflamend is a natural product composed of ten herbal extracts that may be an effective adjuvant against PCa, including CRPC. A common target in cancer treatment is activation of AMPK, a major energy sensor of the cell that inhibits synthesis of macronutrients necessary for cell growth and survival. Zyflamend inhibits CRPC growth, in part, with its ability to upregulate AMPK. This study elucidates Zyflamend's regulation of AMPK activation via two kinases: LKB1 and CaMKK2. LKB1 is a tumor suppressor protein that is reported to be negatively expressed in multiple cancers, including PCa. Upon energetic stress, AMPK undergoes a conformational change that increases its affinity for LBK1 binding and subsequent activation. Unlike LKB1, CaMKK2 is not a tumor suppressor protein, but depends on a rise of intracellular calcium for its activity and regulation of AMPK. Multiple laboratories have reported CaMKK2 is over-expressed in CRPC and acts in a feedback loop with the AR, perhaps the most important regulator of PCa growth. Therefore, inhibiting catalytic activity of CaMKK2 may be a major target to attenuate CRPC growth.

We find Zyflamend concurrently regulates LKB1 and CaMKK2 in the activation of AMPK. Zyflamend-mediated AMPK activation appears LKB1 dependent and simultaneously increases CaMKK2 inhibition, two pathways important in regulating CRPC growth. These results were observed following chemical inhibition of LKB1, siRNA knockdown, and the use of LKB1-null

and KD-LKB1 HeLa cells. In addition, WT-LKB1 transfection in HeLa cells resulted in Zyflamend-stimulated AMPK phosphorylation at its catalytic Thr172 subunit.

On the other side, AMPK activation was not only CaMKK2 independent, but Zyflamend robustly increased a phosphorylation site (Ser511) identified to inhibit its catalytic activity. These results are important since CaMKK2 is reportedly linked to the AR in human CRPC. CaMKK2 phosphorylation and inhibition appears mediated by DAPK, a tumor suppressor protein that regulates apoptosis and autophagy. Overall, to our knowledge, this study is the first to identify a natural product that concurrently regulates LKB1 and CaMKK2 in AMPK activation. These findings elucidates Zyflamend's role as a potential therapeutic adjuvant in the treatment of prostate cancer.

Appendix

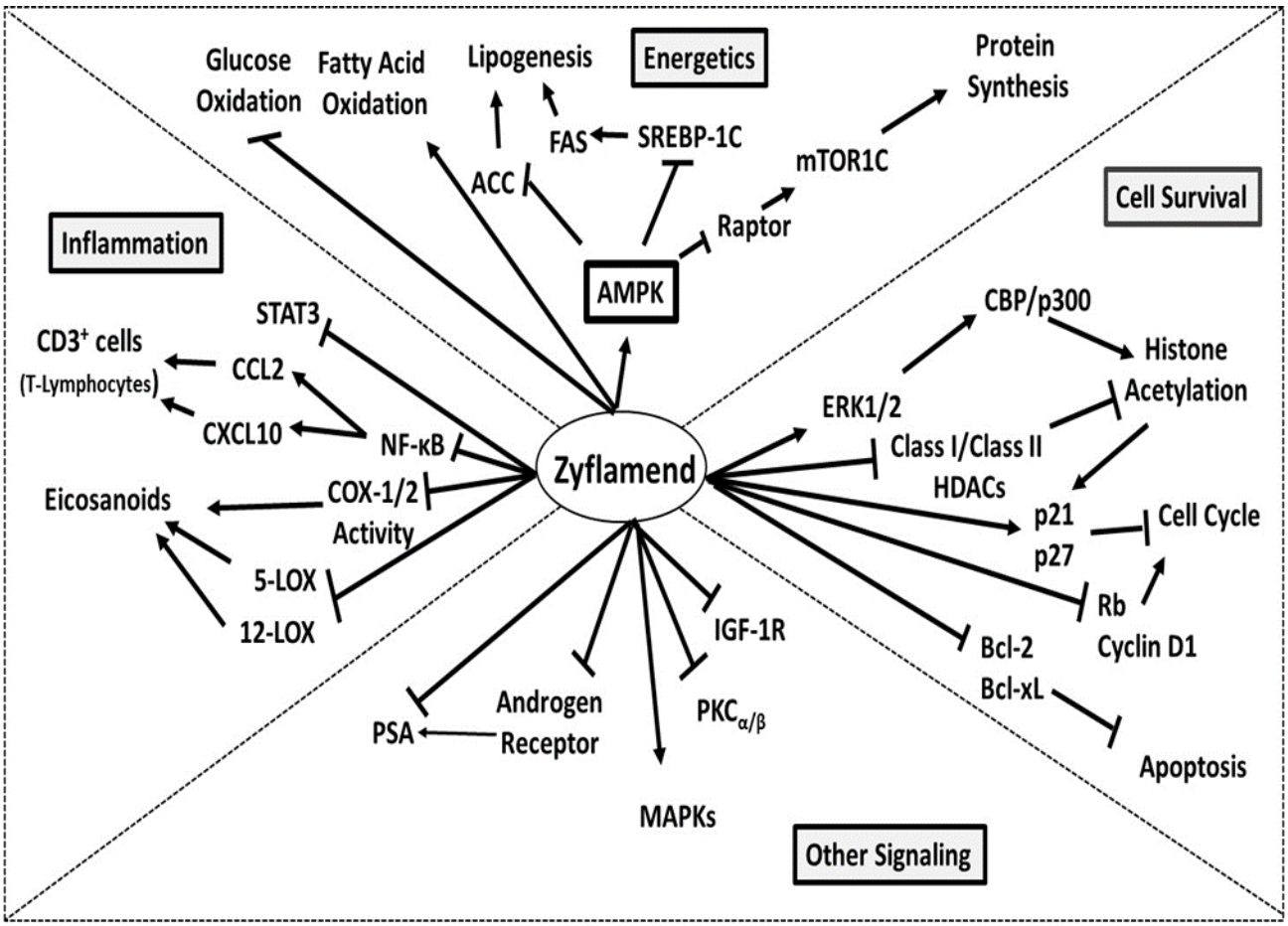

Figure 7. Summary of the effects of Zyflamend on prostate cancer (with permission from reference [3]).

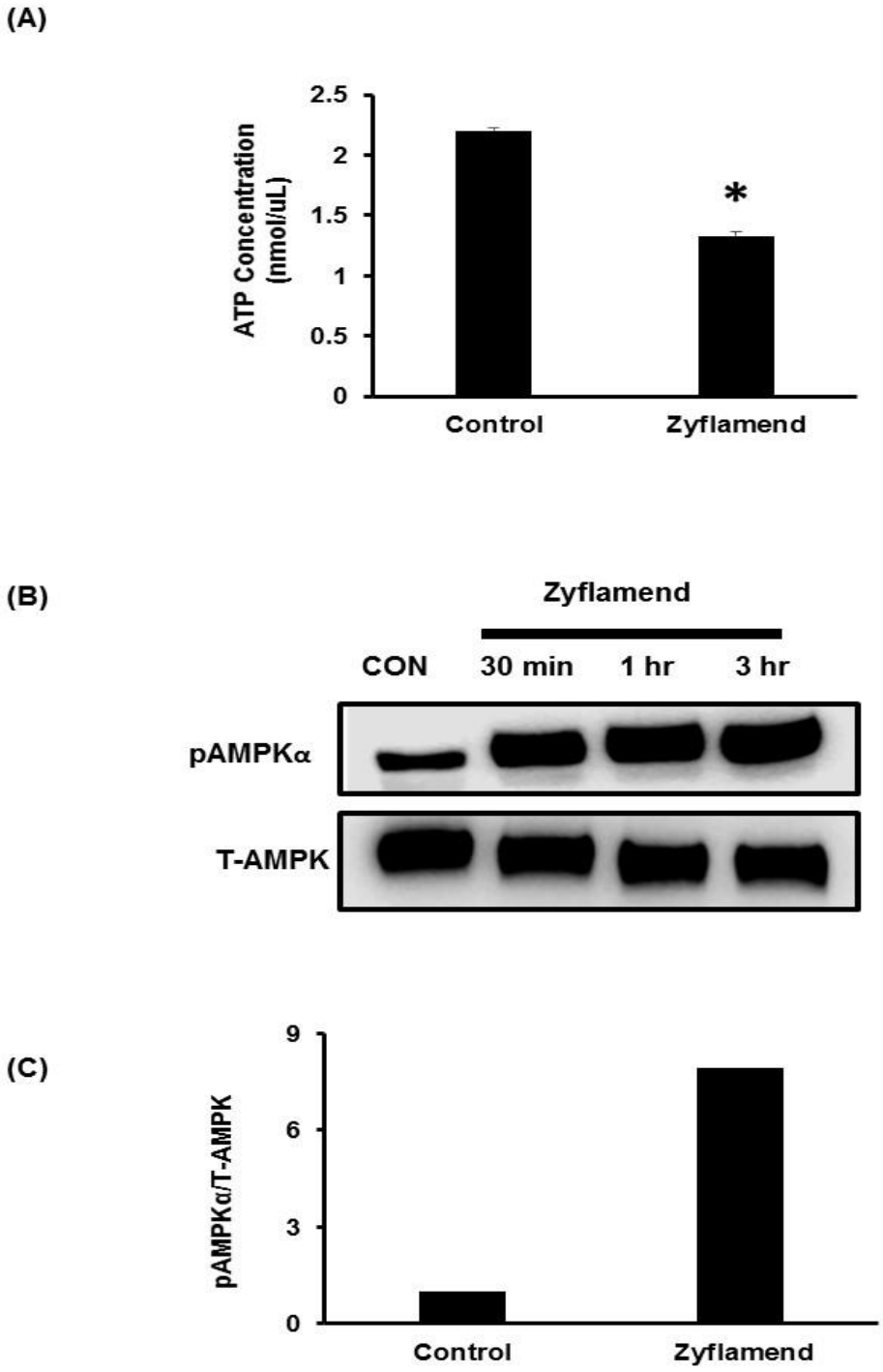

Figure 8. The effects of Zyflamend on cellular ATP levels and phosphorylation of AMPKα (at Thr172) in CWR22Rv1 cells. (A) ATP levels of CWR22Rv1 cells treated in the presence or absence of Zyflamend (200 μg/mL, 30 min). (B, C) The effects of Zyflamend (200 μg/mL, 30 min – 3 hr) on phosphorylation of AMPKα in CWR22Rv1 cells. Data is presented as mean±SEM, n=4. Abbreviations: Con, Control.

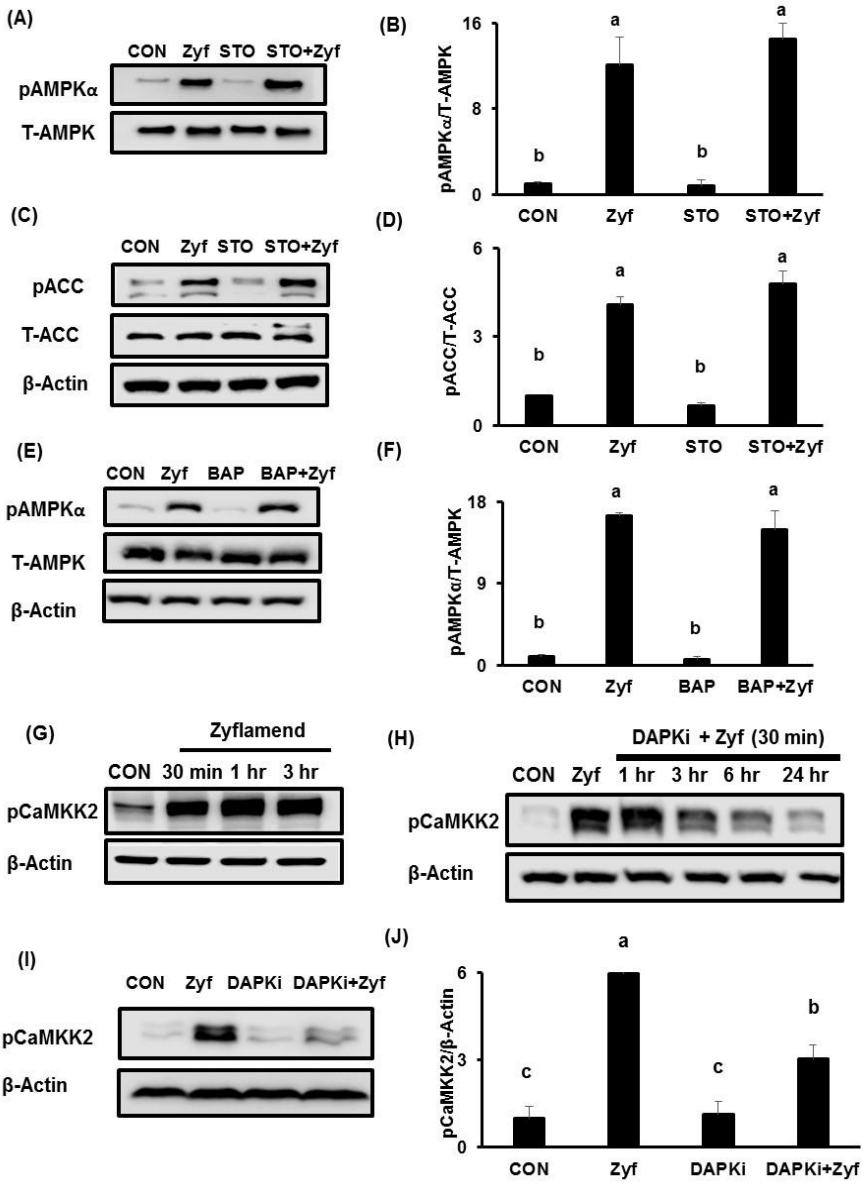

Figure 9. The effects of CaMKK2 inhibition (STO-609) on pAMPKα (at Thr172) and pACC (at Ser79), phosphorylation of CaMKK2 (at Ser511), and inhibition of DAPK with DAPK inhibitor ± Zyflamend in CWR22Rv1 cells. (A-D) Western blot of pAMPKα and pACC following treatment of STO-609 (10 μM, 30 min) ± Zyflamend (200 μg/mL, 30 min). (E, F) Western blot of pAMPKα following treatment of BAPTA-AM (30 μM, 30 min) ± Zyflamend (200 μg/mL, 30 min). (G) Western blot of pCaMKK2 ± Zyflamend (200 μg/mL, 30 min – 3hr). (H) Western blot of pCaMKK2 following treatment of DAPKi (20 μM, 1 hr – 24 hr) in the presence of Zyflamend (200 μg/mL, 30 min). (I, J) Western blot and graph comparison of pCaMKK2 following treatment of DAPKi (20 μM, 24 hr) ± Zyflamend (200 μg/mL, 30 min). Data are presented as mean±SEM, n=3. Bars with different letters are statistically different at $p<0.05$. Abbreviations: BAP, BAPTA-AM; CON, Control; DAPKi, DAPK inhibitor, STO, STO-609; Zyf, Zyflamend.

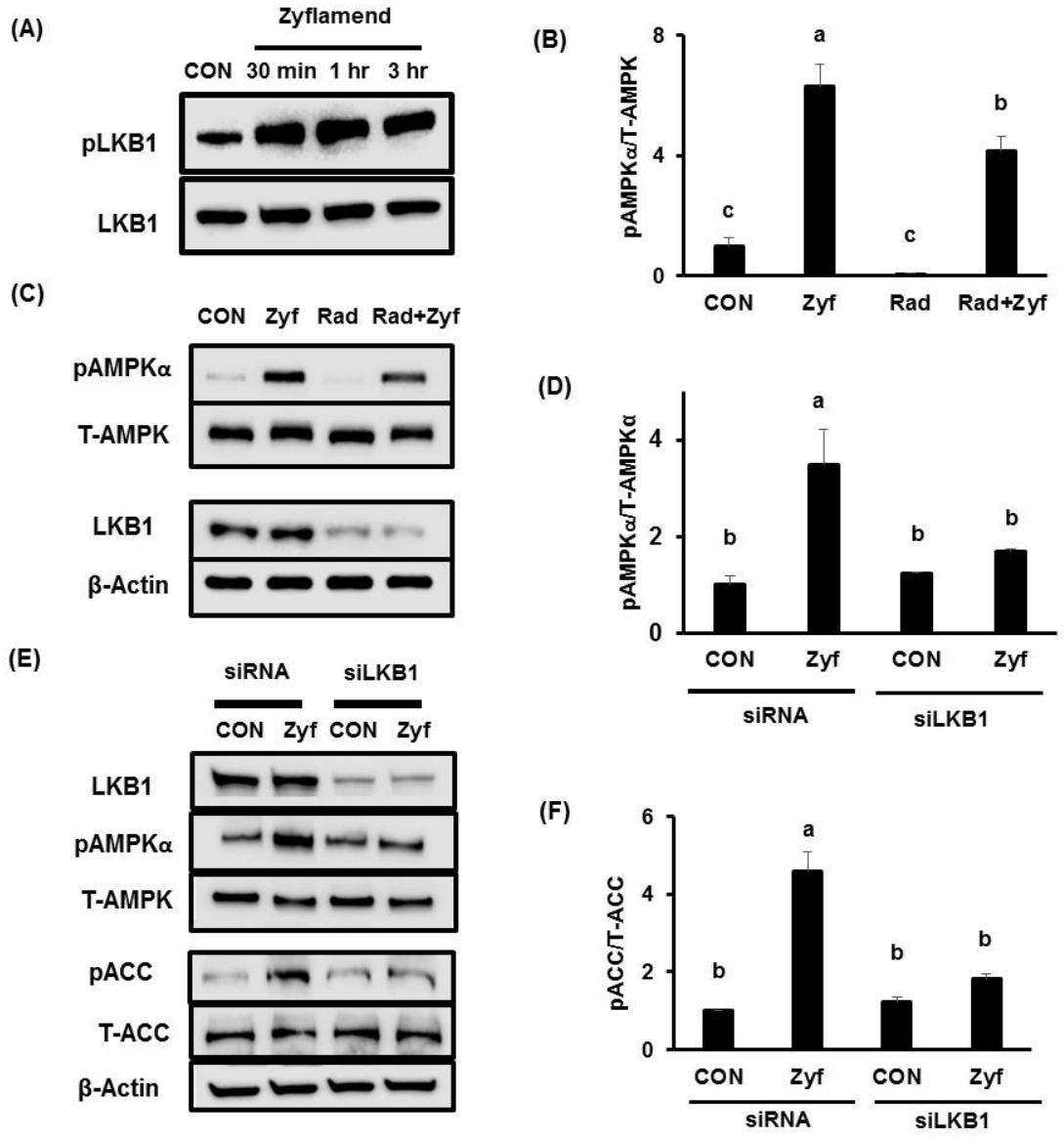

Figure 10. The effects of Zyflamend on phosphorylation of LKB1 (at Ser428) and AMPKα (at Thr172) following knockdown of LKB1 in CWR22Rv1 cells. (A) Western blot of pLKB1 ± Zyflamend (200 μg/mL, 30 min – 3hr). (B, C) Western blot of pAMPKα following treatment with radicicol (5 μM, 24 hr) ± Zyflamend (200 μg/mL, 30 min). (D-F) Western blot of pAMPKα and pACC (at Ser79) following knockdown of LKB1 ± Zyflamend (200 μg/mL, 30 min). Data are presented as mean±SEM, n=3. Bars with different letters are statistically different at $p<0.05$. Abbreviations: CON, Control; Rad, Radicicol; Zyf, Zyflamend.

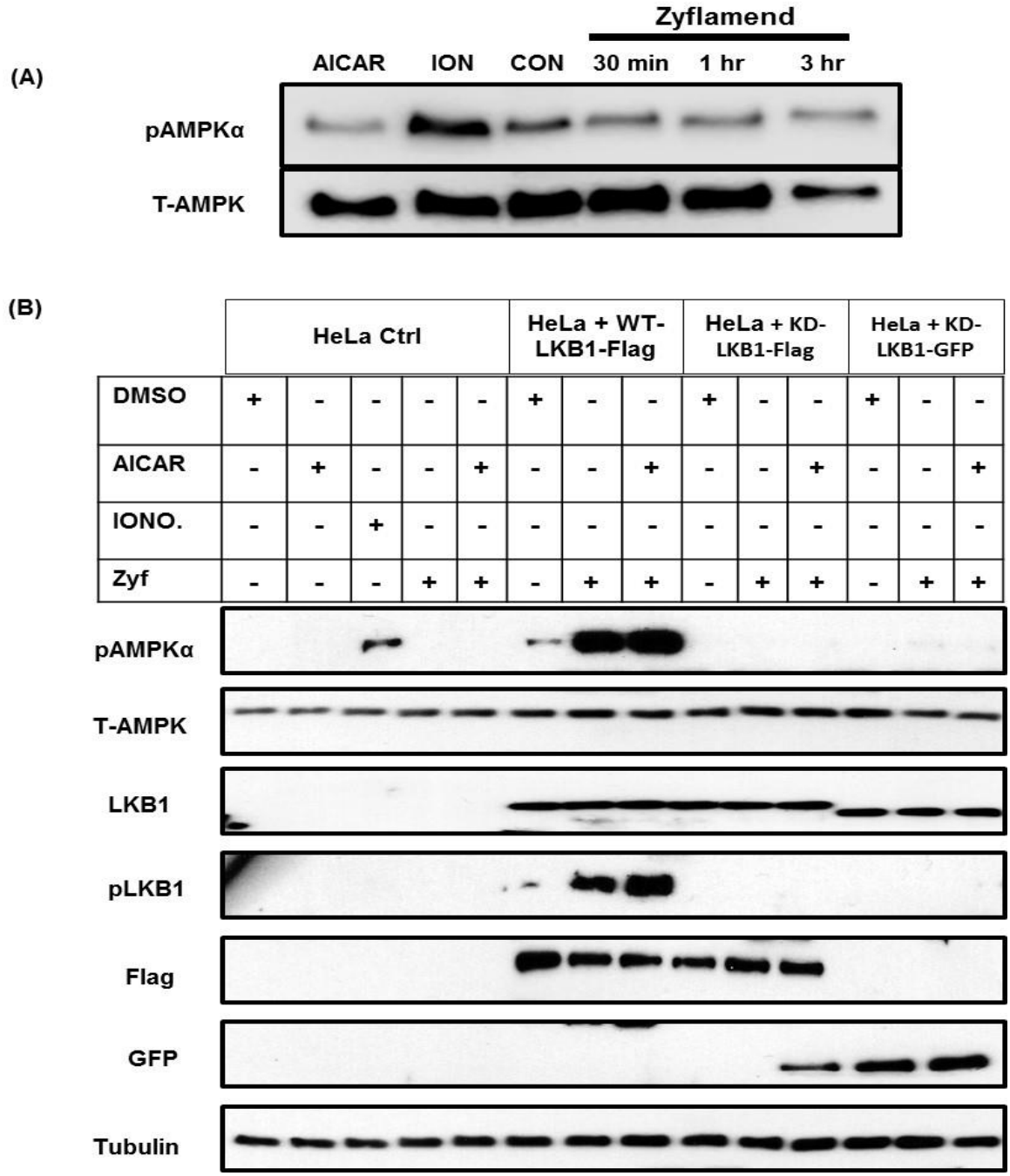

Figure 11. Effects of Zyflamend on pAMPK (at Thr172) and pLKB1 (at Ser428) in HeLa cells null for LKB1, transfected with wild type (WT) LKB1 or with two catalytically dead (KD) mutants of LKB1. (A) Western blot of pAMPK in HeLa cells ± AICAR, (1 mM, 1 hr), ionomycin (1 μM, 1 hr) or Zyflamend (200 μg/mL, 30 min – 3 hr). (B) Western blot of pAMPK in HeLa cells, WT-LKB1 HeLa cells, and KD-LKB1 HeLa cells ± AICAR (1 mM, 1 hr), ionomycin (1 μM, 1 hr), and/or Zyflamend (200 μg/mL, 1 hr). Abbreviations: Con, Control; DMSO, dimethyl sulfoxide; Ion, Ionomycin; Zyf, Zyflamend.

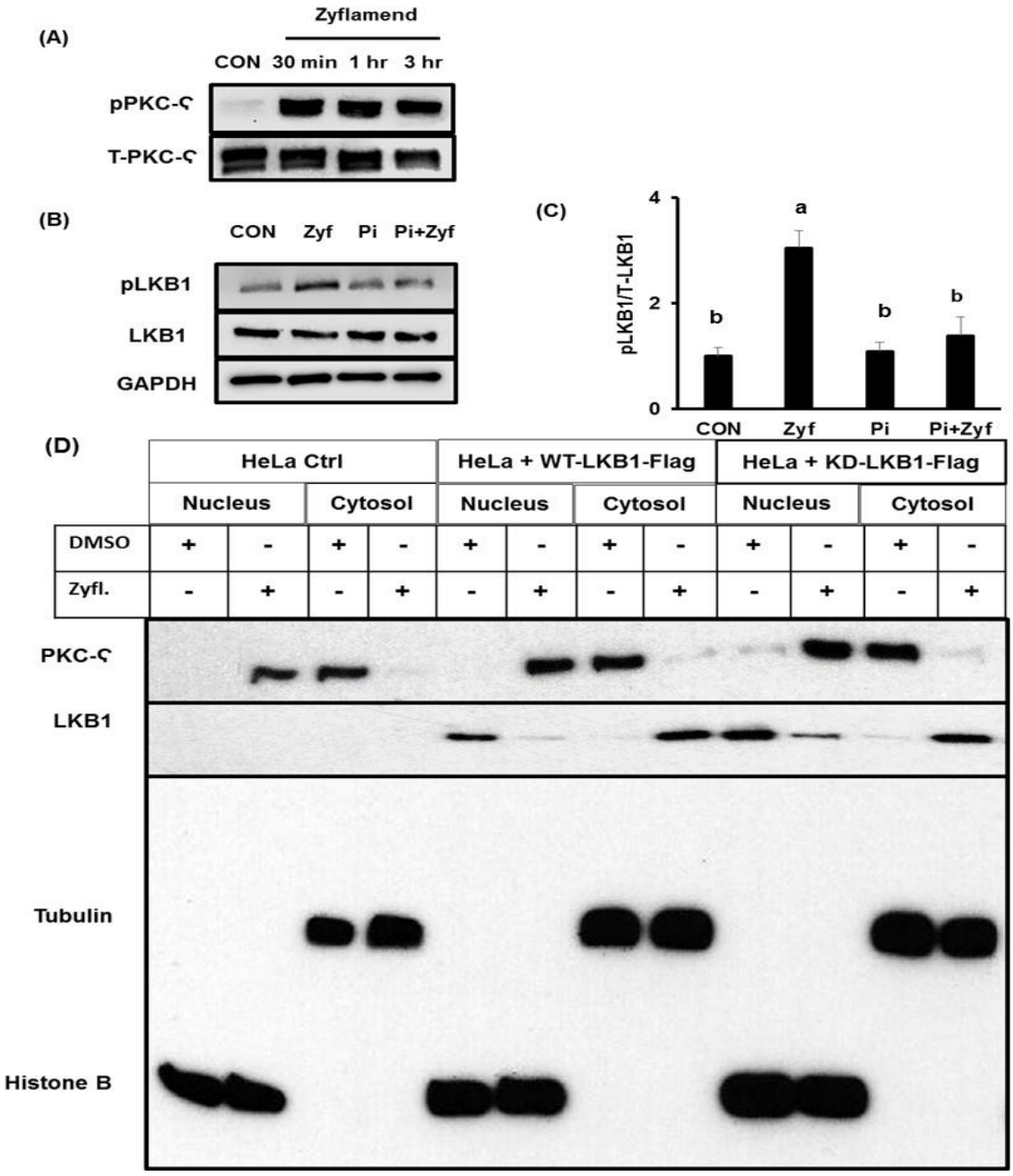

Figure 12. The effects of Zyflamend on phosphorylation of PKCζ (at Thr410) and cellular location of PKCζ and LKB1 in HeLa cells null for LKB1, transfected with wild type (WT) LKB1 or with a catalytically dead (KD) mutant of LKB1. (A) Western blot of phosphorylation of PKCζ ± Zyflamend (200 μg/mL, 30 min – 3 hr). (B,C) Western blot of pLKB1 (at Ser428) following treatment with Zyflamend, PKCζ inhibitor (5 μM, 30 min) or pretreatment with the PKCζ inhibitor plus Zyflamend (200 μg/mL, 30 min). (D) Western blot for PKCζ and LKB1 following subcellular fractionation (cytosol and nucleus) in HeLa cells null for LKB1, transfected with WT LKB1 or with a KD mutant of LKB1 treated with or without Zyflamend (200 μg/mL, 1 hr). Abbreviations: CON, Control; Pi, PKCζ inhibitor; Zyf, Zyflamend.

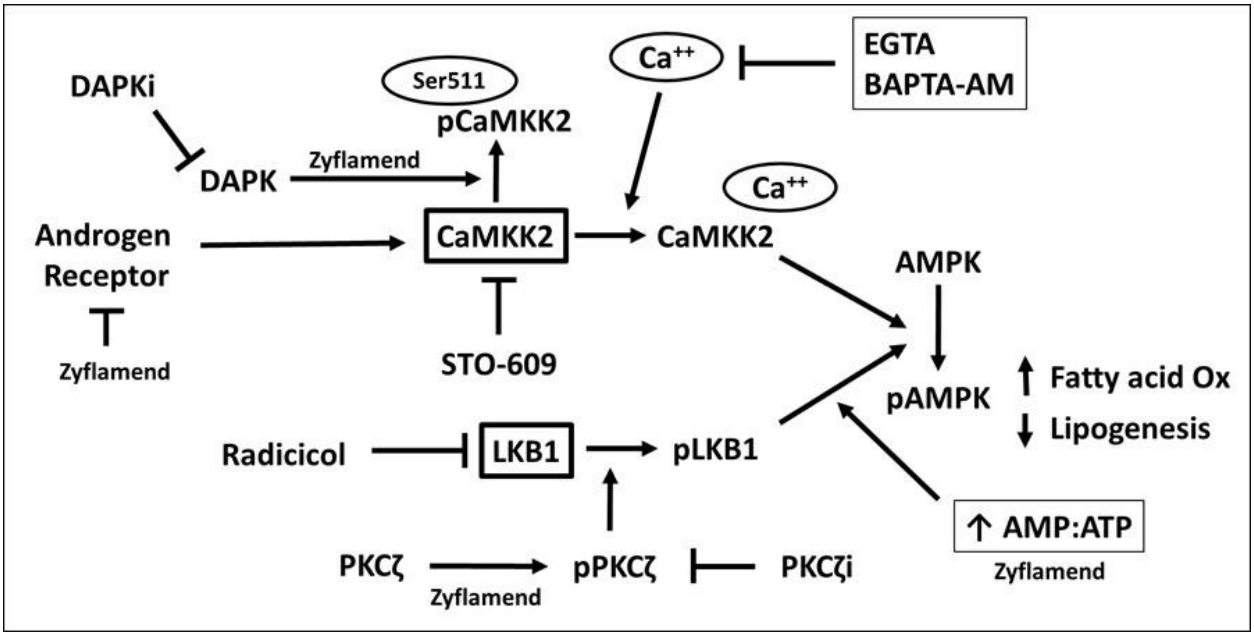

Figure 13. Summary of the effects of Zyflamend on AMPK regulation by signaling pathways of LKB1 and CaMKK2. Zyflamend has been shown to inhibit castrate-resistant prostate cancer, in part, through the activation of AMPK. This activation is mediated by the increase in AMP:ATP ratio and the activation of the tumor suppressor protein LKB1 following phosphorylation by PKCζ. Simultaneously, Zyflamend inhibits the tumor promotor CaMKK2 via phosphorylation at Ser511 by DAPK.

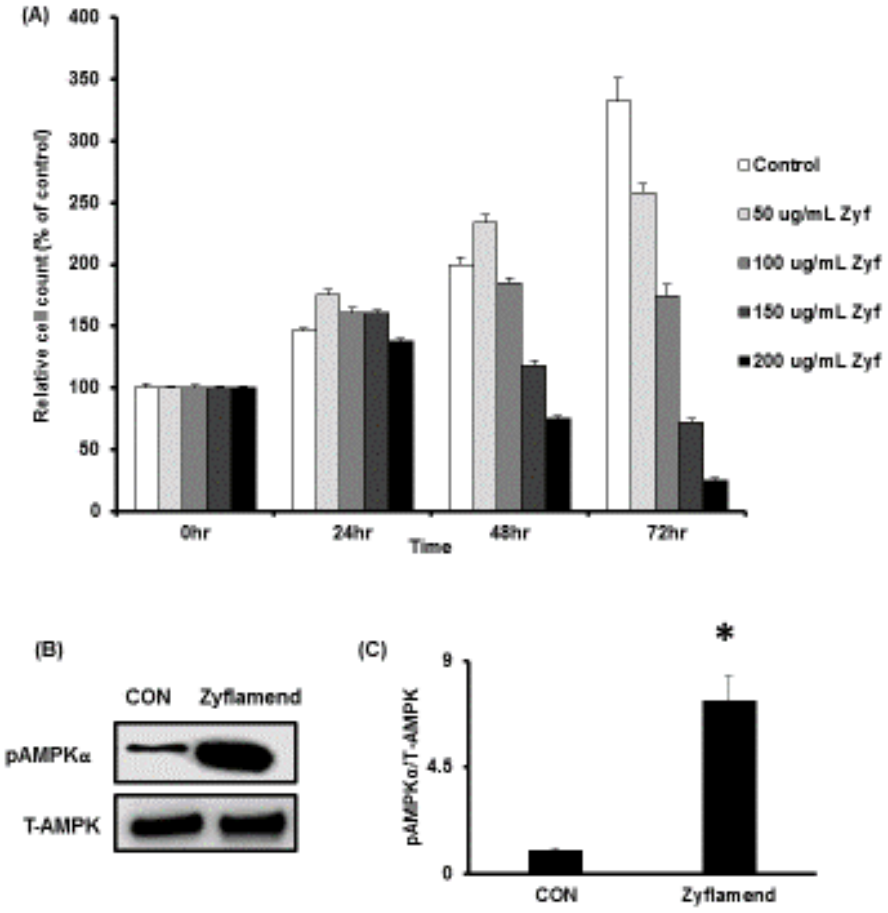

Figure S.1. The effects of Zyflamend on cell proliferation and phosphorylation of AMPKα (at Thr172) in HCT 116 cells. (A) MTT assay of HCT 116 cells in the presence or absence of Zyflamend (50-200 μg/mL, 0 hr – 72 hr). (B, C) The effects of Zyflamend (200 μg/mL, 3 hr) on phosphorylation of AMPKα in HCT 116 cells. Data is presented as mean±SEM, n=8 (A) and n=3 (C) and statistically significant at $p<0.05$. Abbreviations: Con, Control.

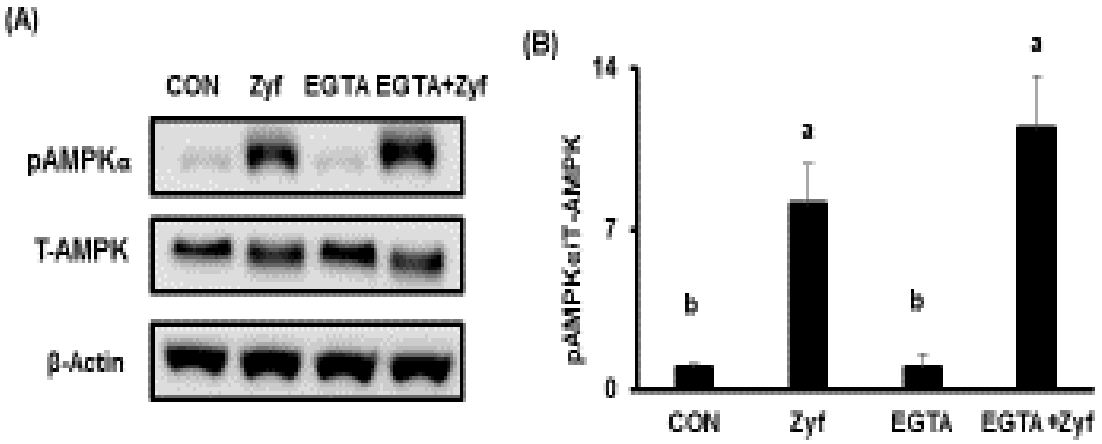

Figure S.2. The effects of CaMKK2 inhibition on pAMPKα ± Zyflamend in CWR22Rv1 cells following pretreatment with the calcium chelator EGTA. (A, B) Western blot of pAMPKα (at Thr172) following treatment of EGTA (2 mM, 30 min) ± Zyflamend (200 μg/mL, 30 min). Data are presented as mean±SEM, n=3. Bars with different letters are statistically different at $p<0.05$. Abbreviations: CON, Control; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N, N, N',N'-tetraacetic acid; Zyf, Zyflamend.

Vita

Amber F. MacDonald was born and raised in Beckley, WV to the parents of Macel Tolbert MacDonald and the late, Jack P. MacDonald on September 11th, 1989. She has three older brothers and three nieces. Amber graduated from Woodrow Wilson High School in 2008 and then attended Bluefield College, Bluefield, VA where she played softball and ran cross-country. In 2012, Amber graduated from Bluefield College, Magna Cum Laude with a Bachelor of Science degree in Exercise and Sports Science concentrating in Sports Medicine. In her free time, Amber enjoys spending time with family and friends, attending church, reading, watching sports, working out, and listening to music. Amber is currently pursuing her Master of Science in Nutrition at the University of Tennessee, Knoxville.

"But without faith it is impossible to please him; for he that cometh to God must believe that he is, and that he is a rewarder of them that diligently seek him."

- **Hebrews 11: 6**

"It's not about how hard you can hit, it's about how hard you can get hit and keep moving forward."

- **Rocky Balboa**

"The journey is the reward."

- **Steve Jobs**