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Zyflamend, a polyherbal mixture, inhibits lipogenesis and mTORC1 signalling via activation of AMPK

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ABSTRACT

A select blend of herbal extracts, Zyflamend, inhibits tumour growth via multiple mechanisms that arise through synergistic interactions between individual components. The objective in this study was to determine if the antiproliferative effects are mediated, in part, through the activation of AMPK signalling and inhibition of its downstream targets, lipogenesis and mTORC1 complex. By increasing the phosphorylation of AMPK in CWR22Rv1 and PC3 prostate cancer cell lines, Zyflamend decreased *de novo* lipid biosynthesis by decreasing the activity of acetyl-CoA carboxylase and the expressions of fatty acid synthase and SREBP-1c, with a concomitant up regulation of fatty acid oxidation. In addition, via AMPK, Zyflamend inhibited the activity of mTORC1 complex by directly phosphorylating raptor. Treatment with AICAR (activator of AMPK), siRNA knockdown, pharmacological inhibition, and overexpression of AMPK were used to confirm the up regulation of AMPK by Zyflamend. These results suggest that Zyflamend inhibited tumour cell proliferation via up regulation of AMPK signalling.

1. Introduction

Herbs and spices have been used to enhance flavouring, colour, aroma and stability of food, and more recently for their beneficial effects in terms of their antioxidant and anti-inflammatory properties (Srinivasan, 2014; Viuda-Martos, Ruiz-Navajas, Fernandez-Lopez, & Perez-Alvarez, 2011). In addition to their functional properties, natural products contain a compendium of biologically active compounds that can promote health and well-being and potentially reduce the risk of disease.

Zyflamend® (New Chapter, Brattleboro, VT, USA) is a blend of 10 herbal extracts (ginger, rosemary, turmeric, Chinese goldthread, holy basil, hu zhang, barberry, oregano, green tea and baikal skullcap) formulated based on their antioxidant and potentially anti-inflammatory properties. To date, there have been 14 research articles investigating the anti-cancer effects

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Abbreviations: AMPK, AMP-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; FASN, fatty acid synthase; SREBP-1c, sterol regulatory element binding protein-1c

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of this blend, covering *in vitro*, preclinical and clinical studies involving the prostate, bone, skin, kidney, breast and pancreas (Bemis, Capodice, Anastasiadis, Katz, & Buttyan, 2005; Burke et al., 2015; Capodice et al., 2009; Ekmekcioglu et al., 2011; Huang, Chen, et al., 2011; Huang, McEntee, & Whelan, 2011; Huang et al., 2014; Kim et al., 2012; Kunnumakkara et al., 2012; Mohebati et al., 2012; Rafailov, Cammack, Stone, & Katz, 2007; Sandur et al., 2007; Yan, Xie, Capodice, & Katz, 2012; Yang et al., 2007, 2008), with prostate being the predominant focus. It has been reported that a number of its constituents act synergistically such that physiological doses are possible (Zhao, Collier, Huang, & Whelan, 2014) and multiple mechanisms of action have been linked in a variety of cancer cell lines by down regulating the androgen receptor (Huang, Chen, et al., 2011; Huang et al., 2014; Yan et al., 2012), insulin-like growth factor-1R (Huang, Chen, et al., 2011; Huang, McEntee, et al. 2011), class I and II HDACs (Huang, Chen, et al., 2011; Huang et al., 2014), COX-1 and COX-2 activities (Bemis et al., 2005; Ekmekcioglu et al., 2011; Kunnumakkara et al., 2012), 12-LOX and Rb protein phosphorylation (Yang et al., 2007), and the up regulation of tumour suppressor genes such as p21 and p27 (Bemis et al., 2005; Huang et al., 2014). Zyflamend has also been shown to modulate NF-κB activation (Burke et al., 2015; Kunnumakkara et al., 2012; Sandur et al., 2007), iNOS (Ekmekcioglu et al., 2011), apoptotic pathways (Ekmekcioglu et al., 2011; Kim et al., 2012; Kunnumakkara et al., 2012), and aryl hydrocarbon receptor expression (Mohebati et al., 2012). It is believed that it is the combination of bioactives that accounts for modulation of multiple pathways at relatively low doses (as described in Huang, Chen, et al., 2011; Zhao et al., 2014).

AMP-activated protein kinase (AMPK) is a master energy sensor in cells and responds to changes in the ratio of AMP : ATP such that when this ratio increases it responds by increasing ATP-generating pathways (i.e., β-oxidation) and concomitantly decreasing anabolic pathways such as lipogenesis and protein synthesis (Hardie, Ross, & Hawley, 2012b). AMPK is a multimeric protein consisting of a catalytic subunit (α) and two regulatory subunits (β and γ) (Hardie, Ross, & Hawley, 2012a; Hardie et al., 2012b). Activation of AMPK involves the phosphorylation of Thr172 of the α-subunit. Up regulation of AMPK activity inhibits acetyl CoA carboxylase (ACC) activity and down regulates the expression of fatty acid synthase (FASN) and the transcription factor sterol regulatory element binding protein-1c (SREBP-1c), key regulators of lipogenesis. AMPK also targets mTORC1, a complex of proteins composed of mammalian target of rapamycin (mTOR), regulatory associated protein of mTOR (raptor), GβL (also known as mLST8) and PRAS40 (Shaw, 2009). This complex promotes protein synthesis and is negatively regulated by AMPK, in part, via the phosphorylation of raptor. Raptor acts as a scaffolding protein for downstream targets of the mTOR complex such as ribosomal S6 kinase (S6K) (Shaw, 2009). Recently, AMPK has been shown to be a potentially important target in the treatment of prostate cancer (Flavin, Zadra, & Loda, 2011; Wright & Stanford, 2009; Xiang, Saha, Wen, Ruderman, & Luo, 2004; Zadra et al., 2014; Zakikhani, Dowling, Sonenberg, & Pollak, 2008).

Recent research using Zyflamend in androgen-dependent and castration-resistant prostate cancer cell lines suggests multiple pathways may be involved in its synergistic mechanism of action (Huang, Chen, et al., 2011; Huang et al., 2014; Zhao et al., 2014). Preliminary evidence from our laboratory that demonstrates Zyflamend down regulates SREBP-1c and FASN transcription and increases fatty acid oxidation suggests it may be regulating AMPK, a new discovery related to this blend of bioactives. Therefore, the objective of this study was to determine if Zyflamend could modulate AMPK activation and its downstream targets, biomarkers of fatty acid synthesis and mTORC1 signalling. These novel results, along with those published previously, can provide a more complete picture as to how blends of natural products may promote health and help to reduce the risk of disease as a result of multiple mechanisms of action.

2. Materials and methods

2.1. *Zyflamend*

Zyflamend® (New Chapter, Brattleboro, VT, USA) is derived from the extracts of ten different commonly consumed herbs (w/w): holy basil (*Ocimum sanctum* 12.8%), turmeric (*Curcuma longa* 14.1%), ginger (*Zingiber officinale* 12.8%), green tea (*Camellia sinensis* 12.8%), rosemary (*Rosmarinus officinalis* 19.2%), hu zhang (*Polygonum cuspidatum* 10.2%), barberry (*Berberis vulgaris* 5.1%), oregano (*Origanum vulgare* 5.1%), baikal skullcap (*Scutellaria baicalensis* 2.5%), and Chinese goldthread (*Coptis chinensis* 5.1%). Detailed description and characterization of the preparation of Zyflamend and quality assurance of the mixture have been described previously in detail (Huang, Chen, et al., 2011).

2.2. *Cell culture*

Human prostate cancer cell lines, CWR22Rv1 and PC3 cells (American Type Culture Collection, Rockville, MD, USA), were initially grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) under an atmosphere of 5% CO_2 at 37 °C. For the experimental treatment, CWR22Rv1 and PC3 cells were then cultured in RPMI 1640 supplemented with 0.5% FBS containing 200 μg/mL Zyflamend reconstituted in dimethyl sulfoxide (DMSO) for cell proliferation assay, mRNA extraction, protein isolation and acetyl-CoA measurements. FBS at a concentration of 0.5% represents an androgen depleted environment. Dose–response curves and time course experiments were performed previously (Huang et al., 2014). HEK293 cells (American Type Culture Collection, Rockville, MD, USA) were grown in DMEM media supplemented with 10% FBS under an atmosphere of 5% CO_2 at 37 °C.

2.3. *Cell proliferation*

Cell growth and proliferation inhibition studies were performed using standard 96 well plates and analysed with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Calbiochem, Darmstadt, Germany) assay following the manufacturer's instructions. CWR22Rv1 cells were plated at a density of 2×10^4 cells per well. After 24 h, the FBS concentration in the cell culture media was reduced to 0.5% FBS and the cells were incubated overnight prior to the addition of the Zyflamend® (200 μg/mL), AICAR (100 μM), Compound C

(5 μM) or recombined adenovirus. After 48 h incubation, cell viability and proliferation were measured via the MTT assay. Briefly, the medium was replaced by 100 μL of 0.5 mg/mL MTT in RPMI1640 media supplemented with 10% FBS and cells were incubated for 4 h at 37 °C. Intracellular formed crystals were solubilized with 100 μL isopropanol/0.04 N HCl. Absorbance was read at 540 nm on a SpectraCount microplate photometer (PerkinElmer Inc, Waltham, MA, USA).

2.4. Down regulation of AMPKα1/2 by small interfering RNA

CWR22Rv1 cells (2×10^5 cells per well in 6-well plates, in transfection medium (sc-36868, Santa Cruz Biotechnology, Dallas, TX) were transfected with 80 pmol of siRNA targeting AMPKα1/2 (sc-29673, Santa Cruz Biotechnology, Dallas, TX, USA) and a scrambled control siRNA (Control siRNA-A, sc-37007, Santa Cruz Biotechnology, Dallas, TX, USA) using transfection reagent (sc-29528, Santa Cruz Biotechnology, Dallas, TX, USA) as described by the manufacturer. Six hours post transfection cells were cultured with RPMI 1640 media containing 10% FBS overnight. After recovery, media were replaced with 0.5% FBS media containing vehicle or Zyflamend (200 μg/mL) for 30 min at 37 °C. The total protein was harvested for Western blot.

2.5. Western blotting

Cells were lysed in RIPA cell lysis buffer (Cell Signaling Technology, Danver, MA, USA). Protein quantification was performed using BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein (20 μg) were fractioned by 8% SDS-PAGE and transferred to a polyvinylidine difluoride (PVDF) membrane by electroblotting. Membranes were blocked by 5% bovine serum albumin (BSA) (Santa Cruz Biotechnology, Dallas, TX, USA) in 0.1% Tris-buffered saline–Tween-20 (TBST) for 1 h at room temperature and incubated in TBST containing primary antibodies overnight at 4 °C. Membranes were incubated with anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) (Cell Signaling Technology, Danver, MA, USA). Protein expression was detected with Pierce ECL Western Blotting detection system and membranes were exposed to Hyperfilm Film (GE Healthcare, Piscataway, NJ, USA). Antibodies against p-AMPKα (p172), AMPKα, p-ACC, ACC, p-Raptor, Raptor, GβL, p-S6K, S6K, and FASN (Cell Signaling Technology, Danver, MA, USA) were used to detect the target protein level. β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) was used as the control. Band intensities were quantified by GelPro® Analyzer 4.0 (Media Cybernetics, Rockville, MD, USA).

2.6. Acetyl-coenzyme A assay

Acetyl CoA was assayed using an acetyl-coenzyme A assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. CWR22Rv1 cells were trypsinized and counted prior to collection. Cells were homogenized in 400 μL acetyl CoA assay buffer and samples were deproteinized by adding 800 μL ice-cold 8% PCA. The resulting supernatant was neutralized by adding 3M $KHCO_3$. Standards were quantified on a 0.2–1 nmol scale and 50 μL of sample was added to each well. The plate was read on a GloMax® Microplate Reader (Promega, Madison, WI, USA) and results were normalized to cell number.

2.7. Construction of recombinant adenovirus

Plasmids containing cDNA of constitutively activated AMPKα1 or AMPKα2 subunits were provided by Dr. Jian Yang (University of Alabama, Tuscaloosa, AL, USA). AMPK cDNA was inserted to pACCMV plasmid and cotransfected with pJM17 into HEK293 cells by Lipofectamine 2000™ transfection reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. Recombinant virus termed ad-CMV-ampkα1/α2 were produced and purified. Virus containing the bacterial β-galactosidase gene (ad-CMV-βGal) was provided by Dr. Guoxun Chen (University of Tennessee, Knoxville, TN, USA) and used as control. Overexpression was verified by western blot.

2.8. RNA isolation and quantitative real time-PCR

Total RNA was isolated from CWR22Rv1 cells using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Contaminating DNA was removed from RNA samples using a Turbo DNA free™ kit (Ambion, Austin, TX, USA). Concentration of total RNA was measured using a NanoDrop 1000™ (Thermo Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) was reverse-transcripted from RNA samples using TaqMan® reagent kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, RNA (2 μg) was mixed with MultiScribe™ Reverse Transcriptase, RNase Inhibitor, dNTP Mixture, random hexamers, RT buffer, and $MgCl_2$ solution and incubated for 10 min at 25 °C, 30 min at 48 °C, 5 min at 95 °C and then stored at 4 °C. cDNA samples were used for quantitative RT-PCR using an ABI Prism® 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). cDNA (0.7 μL) was used as a template for RT-PCR with 2.5 μM primer sets. PCR reactions were performed with a standard thermocycle programme: 1 min at 94 °C, followed by 30 cycles of (15 s at 94 °C; 30 s at 50 °C; 2 min at 72 °C) followed by 5 min at 72 °C. Each sample was run in triplicate and normalized with 36B4. Primers for RT-PCR are listed below:

SREBP1c, F: 5′-GCCATGGATTGCACTTT-3′, R: 5′-CAAGAG AGGAGCTCAATG-3′ FASN, F: 5′-CGCTCGGCATGGCTATCT-3′, R: 5′-CTCGTTGAAGAACGCATCCA-3′ 36B4, F: 5′-TGCATCAGT ACCCCATTCTATCA-3′, R: 5′-AAGGTGTAATCCGTCTCCACAGA-3′. The relative amounts of all mRNAs were calculated using the comparative CT method as previously described with 36B4 as the invariant control (Huang et al., 2014).

2.9. Extracellular flux analysis

The rate of change of dissolved oxygen surrounding the monolayer of CWR22Rv1 cells was measured by an XF24 Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA) following the manufacturer's protocol. Briefly, all assays were conducted using a seeding density of 60,000 cells/well in 500 μL of RPMI 1640 (10% FBS) in a ploy-D-lysine-coated XF24-well microplate (Seahorse Bioscience, Billerica, MA). After culturing cells in a CO_2 incubator for 24 h, the cell medium was changed to Krebs–Henseleit Buffer (KHB, 111 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$,

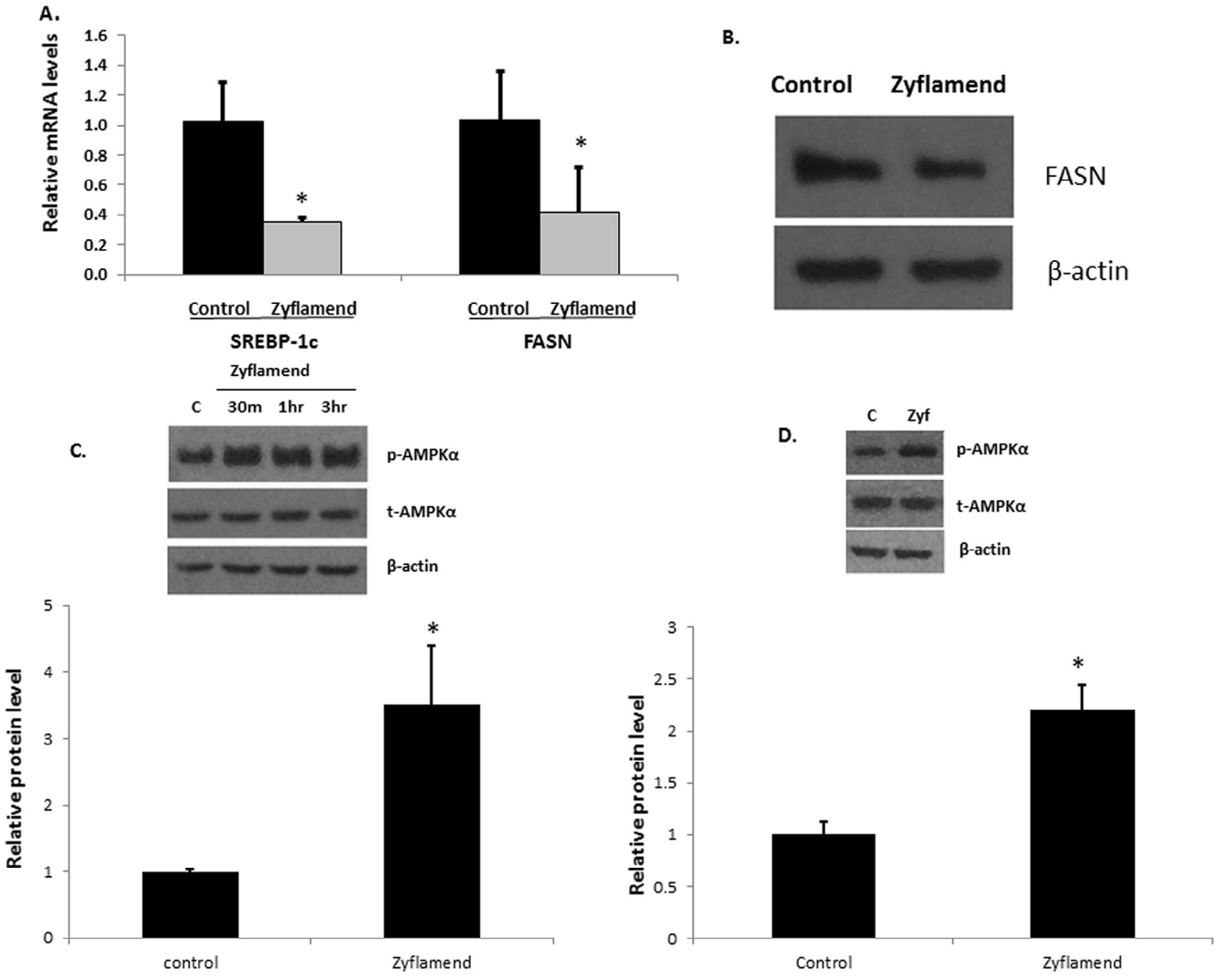

Fig. 1 – The effects of Zyflamend on SREBP-1c and FASN expression and phosphorylation of AMPKα. (A) mRNA levels of SREBP1c and FASN from CWR22Rv1 cells treated in the presence or absence of Zyflamend (200 μg/mL). (B) Western blot for FASN from CWR22Rv1 cells treated with Zyflamend (200 μg/mL). (C, D) The effects of Zyflamend on phosphorylation of AMPKα in CWR22Rv1 and PC3 prostate cancer cell lines. (C) Protein levels of p-AMPKα were determined in CWR22Rv1 cells treated ± Zyflamend (200 μg/mL, 0–3 h) with data (relative ratios of p-AMPK/t-AMPK) summarized at the 30 min time point (bar graph). (D) Protein levels of p-AMPKα (relative ratios of p-AMPK/t-AMPK) were determined in PC3 cells treated ± Zyflamend (200 μg/mL, 30 min). Data are presented as mean ± SD, n = 3. Group comparisons, Zyflamend versus control, * $p < 0.05$.

2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, pH 7.2) supplemented with 2.5 mM glucose, 0.5 mM carnitine and 25 mM HEPES and incubated at 37 °C in a non-CO_2 incubator for 1 h. Then assays were placed in the analyser and the oxygen consumption rate (OCR) could be detected by the fluorescent probes that contain oxygen sensors. In this study, percent change in oxygen consumption rate (%OCR) was determined in CWR22Rv1 cells treated with vehicle (olive oil or DMSO), Zyflamend (200 μg/mL) or AICAR (100 μM) for 1 h. Then, BSA or palmitate was injected and the %OCR was measured over 6 cycles. At the end of the 6 cycles, etomoxir (50 μM), a fatty acid oxidation inhibitor, was injected and %OCR was measured over 3 additional cycles. The total contribution of palmitate oxidation towards OCR (%) was observed after injection of etomoxir. The relative palmitate oxidation was determined by determining the difference before and after etomoxir injection in vehicle groups compared to Zyflamend or AICAR treatment groups.

2.10. *Statistical analysis*

The results are presented as mean ± SD. The data from the extracellular flux analysis are presented as mean ± SEM. For two group comparisons, the data were analysed by two-tailed Student's T-test. For multiple comparisons, the results were analysed by an ANOVA followed by Tukey's post hoc analysis when appropriate. Differences were considered significant at $p < 0.05$.

3. Results

3.1. *Zyflamend inhibited de novo fatty acid synthesis in CWR22Rv1 cells*

In preliminary experiments using CWR22Rv1 cells, Zyflamend treatment (24 h) significantly decreased mRNA levels of FASN and SREBP-1c, the major transcriptional regulator of enzymes in lipogenic pathways (including FASN), by ~60% (Fig. 1A). The effect of Zyflamend on the protein level of FASN (24 h) is presented in Fig. 1B.

3.2. *Zyflamend regulates AMPK phosphorylation in CWR22Rv1 and PC3 prostate cancer cell lines*

In CWR22Rv1 cells, phosphorylation of AMPKα (T172) was increased by Zyflamend (200 μg/mL) ~ 3.5 fold after 30 min treatment (Fig. 1C). Similarly, Zyflamend increased the phosphorylation of AMPKα (T172) by ~2 fold in PC3 cells (Fig. 1D).

3.3. *Zyflamend regulates downstream targets of AMPK*

ACC and mTORC1 complex are two major downstream targets of AMPK. In CWR22Rv1 cells, Zyflamend (200 μg/mL) increased phosphorylation of ACC ~2 fold after 30 min treatment (Fig. 2A, B). The cellular levels of acetyl CoA significantly increased by about 1.5 fold after 24 h treatment of Zyflamend (Fig. 2C).

Regarding mTORC1 complex which includes raptor and GβL (Kim et al., 2003), Zyflamend treatment increased phosphorylation of raptor ~4 fold after 30 min treatment (Fig. 2A, B). Phosphorylation of S6K, a downstream biomarker for activity of mTORC1 complex, was reduced in a time dependent manner (0.5–3 h) with Zyflamend treatment (Fig. 2A, B). In the presence of Zyflamend, GβL protein levels decreased in a time dependent manner (Fig. 2A, B). Increases in the phosphorylation of ACC and raptor were replicated in CWR22Rv1 cells following treatment with the positive control, AICAR (Fig. 3A, B), and in PC3 cells following treatment with Zyflamend (Fig. 3C, D).

To confirm that Zyflamend increased phosphorylation of ACC and raptor by increasing the activity of AMPK, we knocked down AMPKα1/2 subunit by siRNA. Transient knockdown by siRNA transfection resulted in a 50% reduction of AMPKα protein levels (Fig. 4A, B). Concomitantly, phosphorylation of ACC and raptor was decreased by about 50% after AMPKα knockdown. The addition of Zyflamend attenuated the effects of siRNA knockdown of AMPKα1/2 on phosphorylation of ACC and raptor (Fig. 4A, C, D), but did not attain those of Zyflamend-treated controls.

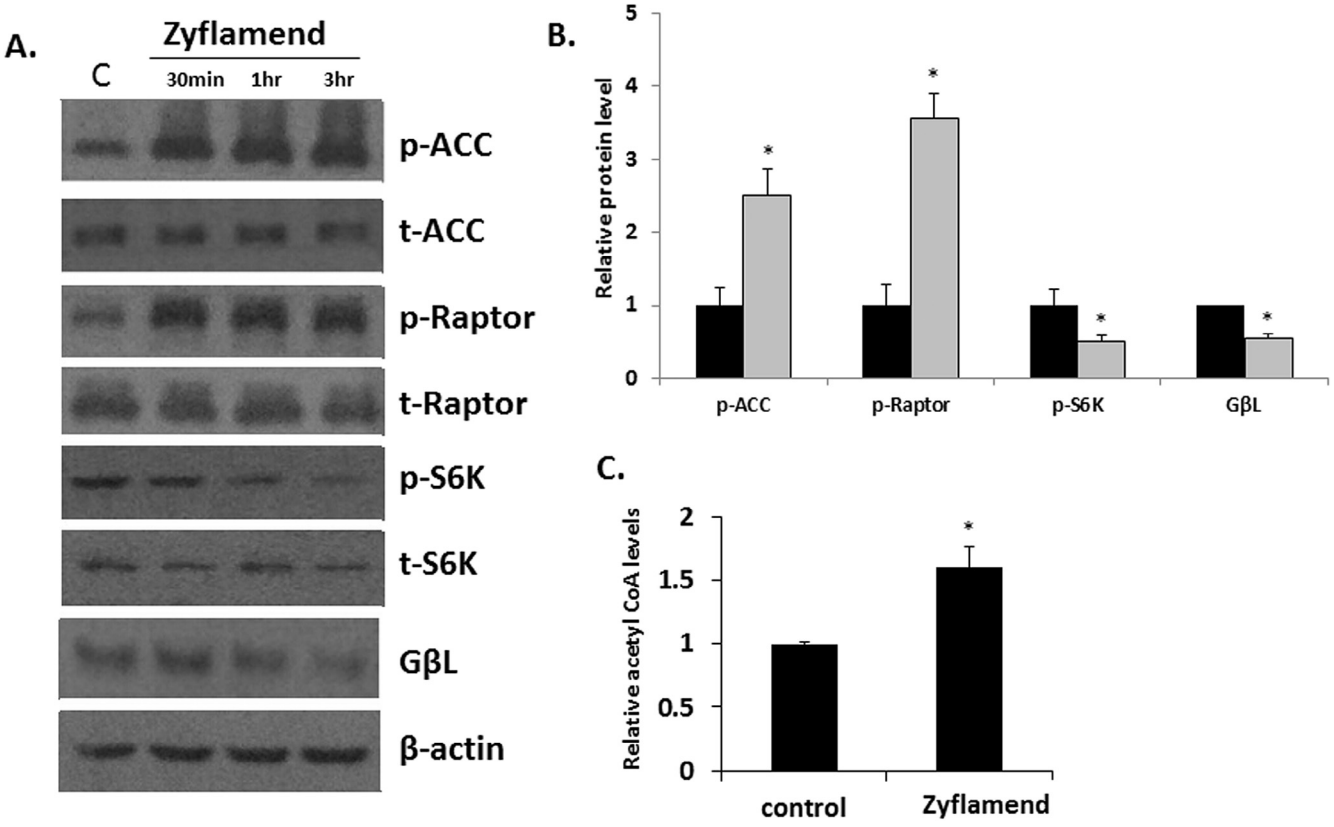

Fig. 2 – Regulation of downstream targets of AMPK by ±Zyflamend in CWR22Rv1 cells. (A, B) The effects of Zyflamend treatment (0.5–3 h) on phosphorylation of ACC, raptor and S6K (relative to total ACC, total raptor and total S6K, respectively) and protein levels of GβL. Data are presented as mean ± SD, n = 3. Group comparisons, Zyflamend versus control, * $p < 0.05$. (C) Effects of Zyflamend on total cellular levels of acetyl CoA. Data are presented as mean ± SD, n = 3. Group comparisons, Zyflamend versus control, * $p < 0.05$.

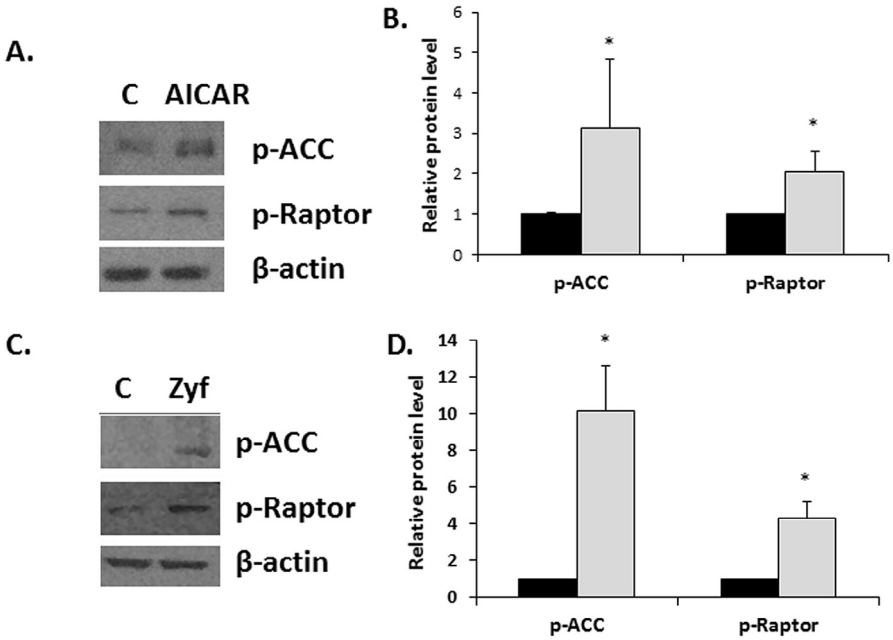

Fig. 3 – Regulation of downstream targets of AMPK by AICAR in CWR22Rv1 cells and Zyflamend (Zyf) in PC3 cells. (A, B) The effects of AICAR (positive control) on phosphorylation of ACC and raptor in CWR22Rv1 cells (black bar: without AICAR; grey bar: with AICAR). (C, D) The effects of Zyflamend on phosphorylation of ACC and raptor in PC3 cells (black bar: without Zyflamend; grey bar: with Zyflamend). Data are presented as mean ± SD, n = 3. Group comparisons, AICAR or Zyflamend versus control,* $p < 0.05$.

3.4. *Zyflamend increased fatty acid oxidation in CWR22Rv1 cells*

As a consequence of activation of AMPK and inactivation of ACC, we next sought to determine if Zyflamend increases fatty acid oxidation. Towards this end, we used an XF24 Flux Analyzer (Seahorse Biosciences) to assess palmitate oxidation in CWR22Rv1 cells treated with Zyflamend or vehicle control. The change in the oxygen consumption rate (OCR) from baseline increased in CWR22Rv1 cells treated with Zyflamend compared to the olive oil vehicle control (Fig. 5A). This effect was negated after injection of etomoxir, an inhibitor of fatty acid oxidation. The difference in OCR before and after etomoxir injection was used to calculate the relative palmitate oxidation, which was approximately 4-fold higher in the Zyflamend group as compared to control (Fig. 5B). As a comparison, the AMPK activator AICAR also stimulated palmitate oxidation to a similar extent as that of Zyflamend (Fig. 5C, D).

3.5. *Zyflamend decreased CWR22Rv1 cell viability, in part, through the activation of AMPK*

Inhibiting AMPK activity by compound C modestly enhanced CWR22Rv1 cell viability after 48 h treatment (Fig. 6A). Zyflamend decreased cell viability and this effect was partially attenuated with the concomitant treatment of compound C (Fig. 6A). To mimic the effect of Zyflamend's induction of phosphorylation of AMPKα, cells were treated with AICAR, a known AMPK activator. AICAR (100 μM) treatment decreased cell viability by about 20% after 48 h treatment (Fig. 6C). Treatment with AICAR plus Zyflamend resulted in an additional decrease in cell viability (~30%) (Fig. 6C). Overexpression of AMPKα1/2 subunits (as confirmed by western blot, Fig. 6B) inhibited cell viability in CWR22Rv1 cells by 40% (Fig. 6C), and this effect was augmented in the presence of Zyflamend.

4. Discussion

Zyflamend is a commercial product composed of the extracts of ten herbs. Its original formulation almost 2 decades ago was based on those herbs that had antioxidant and anti-inflammatory properties. Its subsequent use in experimental investigations in an assortment of cancer models has drawn attention by a variety of laboratories interested in the role of natural products in health and disease (Bemis et al., 2005; Burke et al., 2015; Capodice et al., 2009; Ekmekcioglu et al., 2011; Huang, Chen, et al., 2011; Huang, McEntee, et al. 2011; Huang et al., 2014; Kim et al., 2012; Kunnumakkara et al., 2012; Mohebati et al., 2012; Rafailov et al., 2007; Sandur et al., 2007; Yan et al., 2012; Yang et al., 2007, 2008).

There are always concerns regarding reproducibility and quality control of commercial products. The quality control in the preparation of these extracts has been described in detail elsewhere (Huang, Chen, et al., 2011). Assurance of that quality control has been verified by the corroborative reproducible results generated at different times, with different lots, in multiple laboratories, under different circumstances, using similar and different cell lines with similar concentrations (Bemis et al., 2005; Burke et al., 2015; Capodice et al., 2009; Ekmekcioglu et al., 2011; Huang, Chen, et al., 2011; Huang, McEntee, et al. 2011;

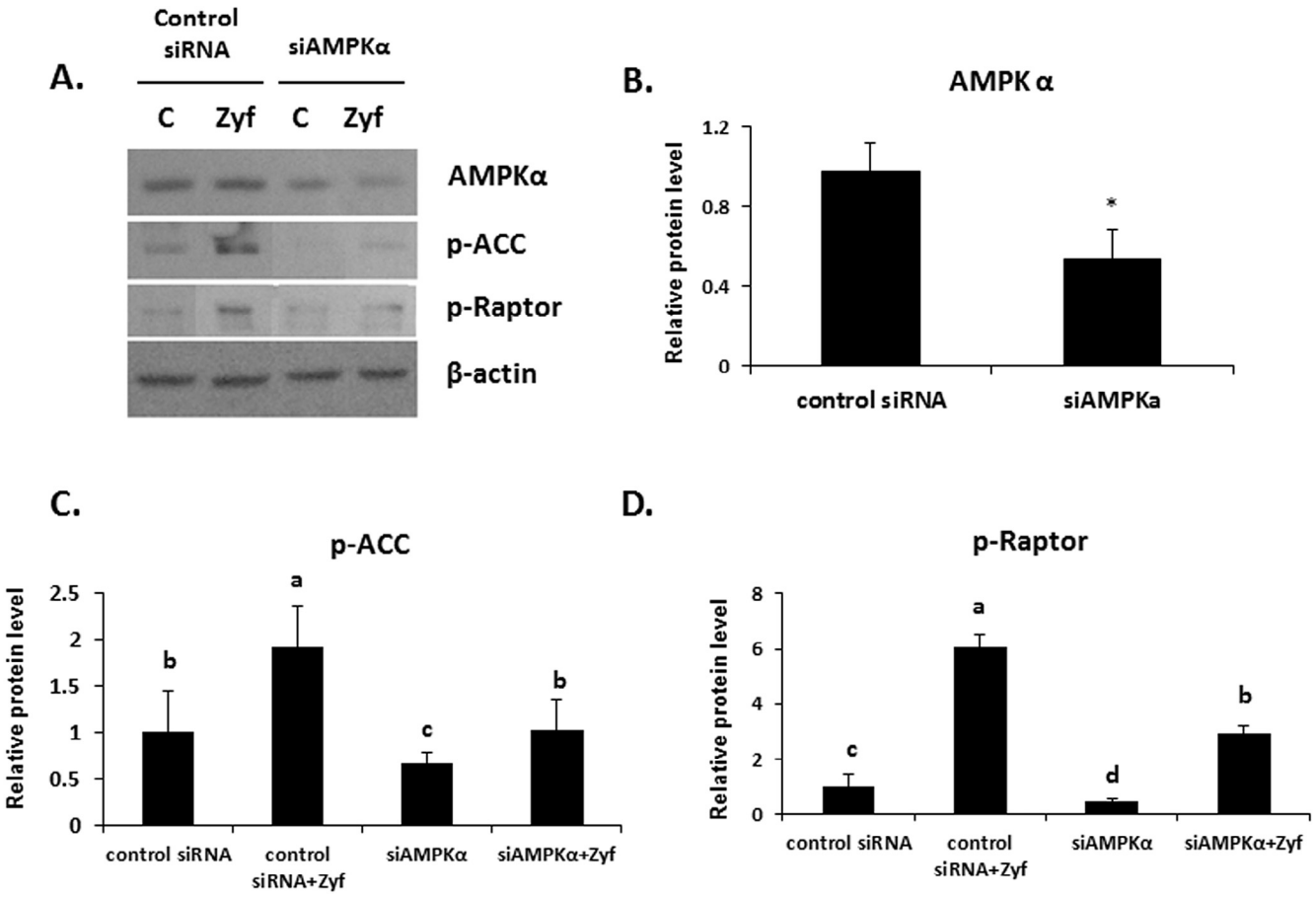

Fig. 4 – The effects of partial knockdown of AMPKα1/2 on phosphorylation of ACC and raptor in the presence or absence of Zyflamend. (A, C, D) Western blot of p-ACC and p-raptor following knockdown of AMPKα1/2 in the presence or absence of Zyflamend. Data are presented as mean ± SD, n = 3. [a,b,c,d]Bars with different letters within each figure are significantly different at $p < 0.05$; (B) Group comparison, siAMPKα versus control,* $p < 0.05$.

Huang et al., 2014; Kim et al., 2012; Kunnumakkara et al., 2012; Mohebati et al., 2012; Rafailov et al., 2007; Sandur et al., 2007; Yan et al., 2012; Yang et al., 2007, 2008). The best combinations of extracts within this formulation have not been systematically determined because it would be infeasible to reproduce the results from 1028 possible combinations. However, recent evidence using biologic and molecular endpoints suggests that the compendium of compounds found in individual extracts is synergistically more efficacious than isolated bioactives derived from those extracts and that the combinations of extracts are synergistically more efficacious than the extracts used individually (Zhao et al., 2014). These combinations are more likely to have bioactivity at concentrations that would be more physiologically relevant (Zhao et al., 2014). Furthermore, the use of well-defined extracts in testing mechanisms of action provides evidence more related to how people eat (*viz.* people eat foods with their complexity of bioactives, not purified compounds from those foods). As such, generating mechanisms of action using well-defined combinations should further the understanding of the food–health relationship.

In our laboratory, we have been studying the effects of Zyflamend on preclinical models of chronic diseases, including cancer (Huang, Chen, et al., 2011; Huang, McEntee, et al. 2011; Huang et al., 2014) and diabetes (Burke et al., 2015), with an emphasis on castration-resistant prostate cancer (Huang, Chen, et al., 2011; Huang, McEntee, et al. 2011; Huang et al., 2014) using cell lines derived from the CWR22 lineage (Nagabhushan et al., 1996; Pretlow et al., 1993). We focus our attention on this lineage because the androgen dependent CWR22 cells can relapse into the androgen-insensitive CWR22R cell line *in vivo* following androgen deprivation (McEntee et al., 2008; Nagabhushan et al., 1996). These cells, unlike the androgen independent PC3 cell line, have a functional androgen receptor and produce prostate specific antigen, common characteristics in human castration-resistant prostate cancer.

Our data pertaining to Zyflamend and several studies from other laboratories (Bemis et al., 2005; Burke et al., 2015; Capodice et al., 2009; Ekmekcioglu et al., 2011; Huang, Chen, et al., 2011; Huang, McEntee, et al. 2011; Huang et al., 2014; Kim et al., 2012; Kunnumakkara et al., 2012; Mohebati et al., 2012; Rafailov et al., 2007; Sandur et al., 2007; Yan et al., 2012; Yang et al., 2007, 2008) reveal diverse mechanisms of action, possibly explaining its robust effects using *in vivo* models of tumorigenesis at oral doses that mimic human intakes (Huang, Chen, et al., 2011; Kunnumakkara et al., 2012). Because herbal extracts are composed of multiple compounds, it is difficult to directly extrapolate a standard concentration. The concentration of

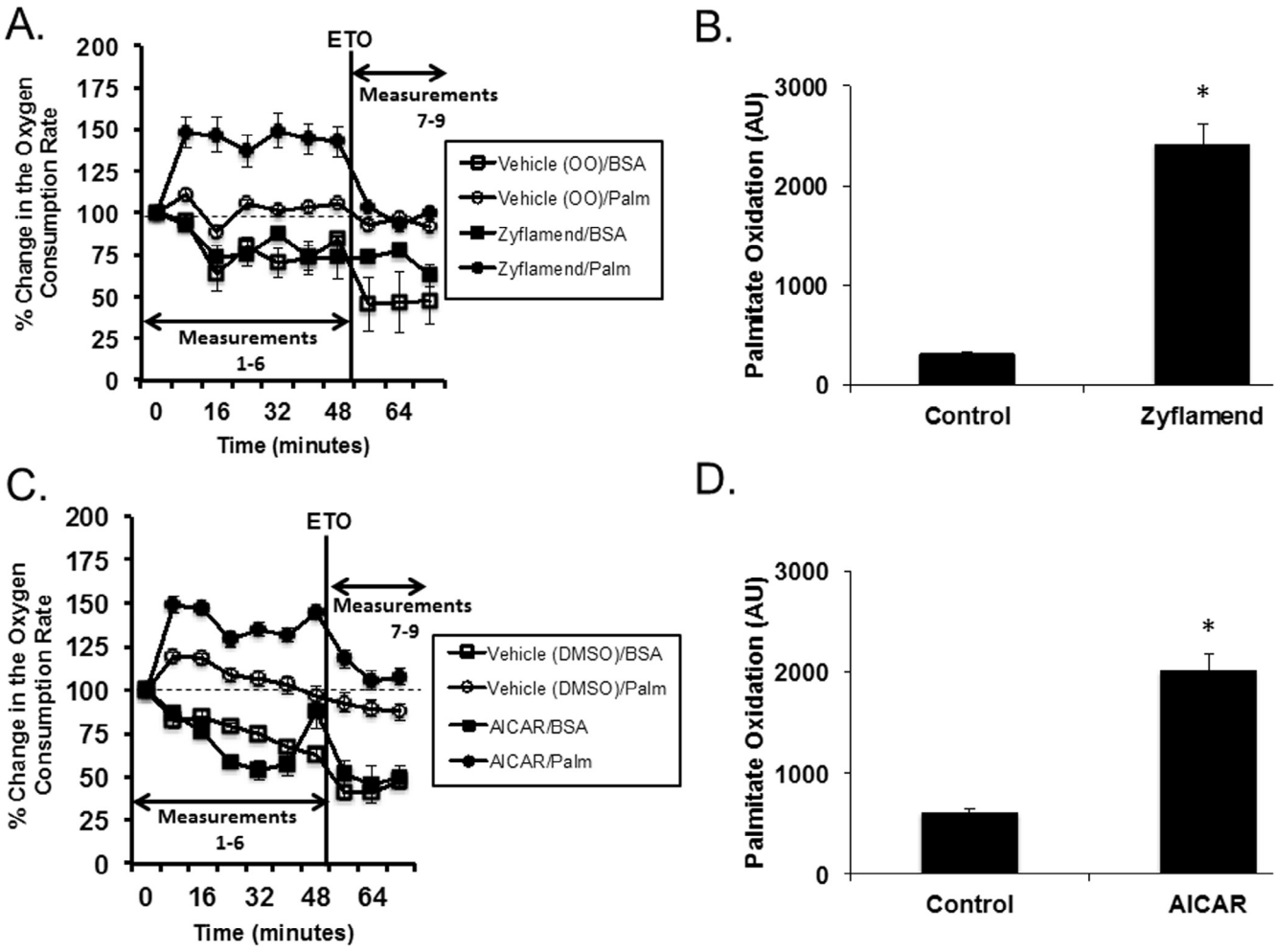

Fig. 5 – The effect of Zyflamend and AICAR on palmitate oxidation in CWR22Rv1 cells. Percent change in oxygen consumption rate (%OCR) in CWR22Rv1 cells treated with vehicle (olive oil or DMSO), Zyflamend or AICAR for 1 h. Then, BSA or palmitate was injected and the %OCR was measured over 6 cycles. At the end of the 6 cycles, etomoxir (50 μM), a fatty acid oxidation inhibitor, was injected and %OCR was measured over 3 additional cycles. The total contribution of palmitate oxidation towards %OCR is observed after injection of etomoxir (A, C). The relative palmitate oxidation was determined by determining the difference before and after etomoxir injection in vehicle groups compared to Zyflamend or AICAR treatment groups. Data points for panels B and D represent the average OCR (%) over 3–5 replicates per experimental condition. Error bars represent ±SEM. [Abbreviations: AU, arbitrary units; ETO, etomoxir (carnitine palmitoyl transferase-1 inhibitor)]. AU – area under the curve after injection of palmitate for each treatment and normalized to etomoxir treatment.

Zyflamend used in this study was based on the physiological achievable concentration of curcumin. The maximum dose of Zyflamend (200 μg/mL) was chosen based on the levels of curcumoids found in the polymixture (~7% w/w in turmeric) (Braga, Leal, Carvalho, & Meireles, 2003) and this was matched to the highest identifiable levels reported in human plasma concentrations (~2 μM) following an oral supplementation of 8 g/d over a 3 month period (Cheng et al., 2001). It has been suggested that these levels *in vitro* could have human physiological relevance (Howells et al., 2007); however, the results generated in this study need to be investigated *in vivo* for confirmation.

The objective of this study was to follow up on previous studies and demonstrate that Zyflamend inhibits lipogenesis by down regulating SREBP-1c and FASN expression and enhancing fatty acid oxidation. To support the rapid proliferation of cancer cells, up regulation of *de novo* fatty acid synthesis is commonly found in solid tumours, including prostate, and this metabolic shift supporting anabolic processes can be the result of dysregulation of AMPK (Flavin et al., 2011; Zadra et al., 2014). Therefore, we logically investigated the effect of Zyflamend on AMPK activation as determined by modulation of its well-accepted downstream targets involved in lipogenesis and mTORC1 signalling. Regulation of AMPK has become an important target for cancer treatment. AMPK activators, such as MT63-78, metformin and AICAR, have been shown to be inhibitors of prostate cancer in a variety of preclinical models (Xiang et al., 2004; Zadra et al., 2014).

Zyflamend increased AMPK activity by increasing phosphorylation of Thr172 of the catalytic subunit, resulting in inhibition of fatty acid synthesis, in part, via the phosphorylation of ACC. Importantly, *de novo* phospholipid biosynthesis

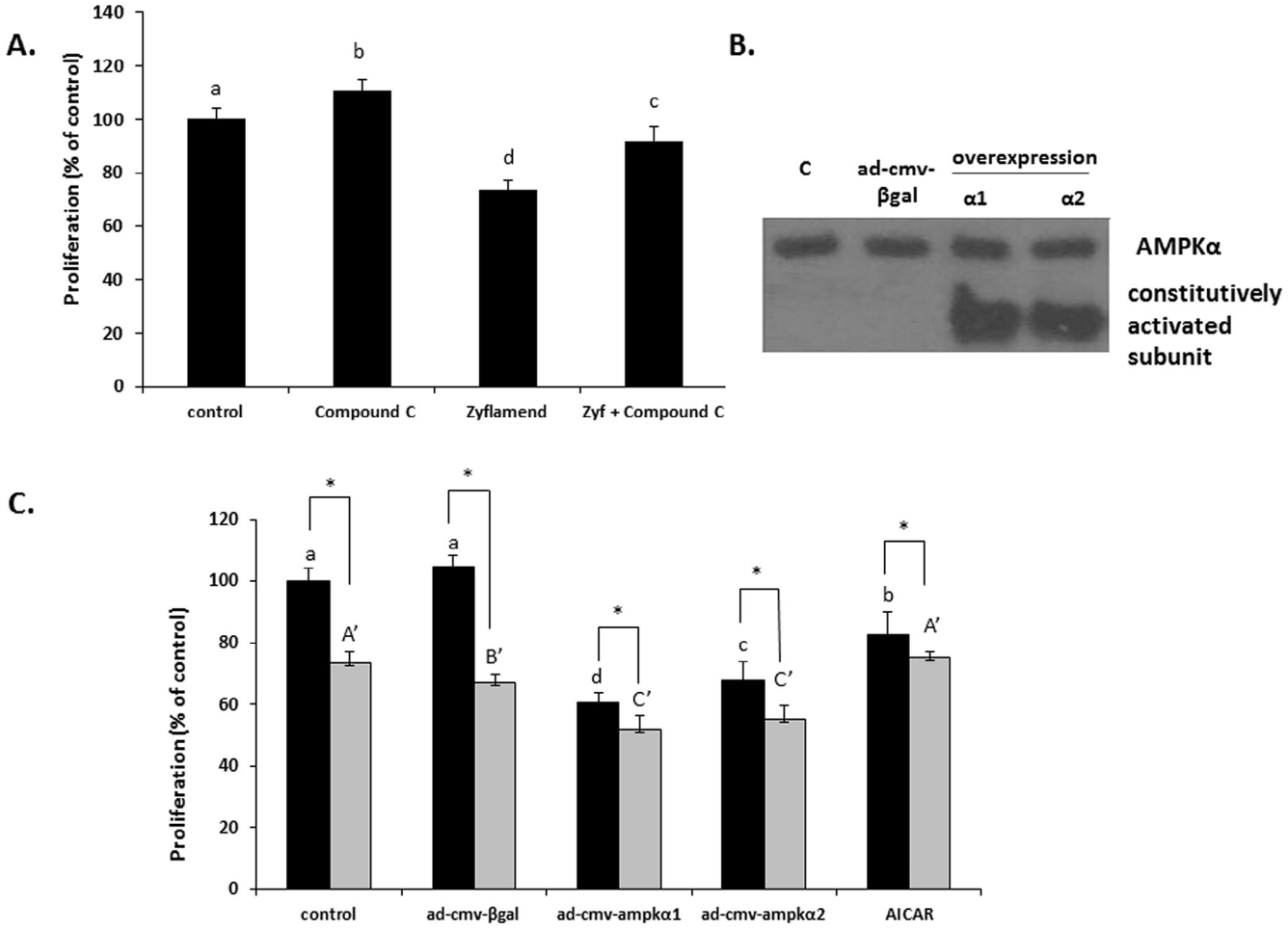

Fig. 6 – The effects on modulating AMPK activity on cell viability with the AMPK inhibitor compound C and overexpression of AMPKα1/2 subunits in the presence or absence of Zyflamend in CWR22Rv1 cells. (A) The effects of cell viability following treatment with compound C, in the presence or absence of Zyflamend. Data are presented as mean ± SD, n = 8. ^{a,b,c,d}Bars with different letters are significantly different at $p < 0.05$. (B) Overexpression by adenovirus as confirmed by western blot. (C) The effects of overexpression of AMPKα1/2 subunits on cell viability in the presence (black bar) or absence (grey bar) of Zyflamend. Data are presented as mean ± SD, n = 8. Bars with different letters (^{a,b,c,d} or $^{A',B',C'}$) are significantly different at $p < 0.05$ (^{a,b,c,d} represent statistical comparisons of the black bars, and $^{A',B',C'}$ represent statistical comparisons of the grey bars). Within group comparisons ±Zyflamend, * $p < 0.05$.

has been shown to be a primary target for fatty acids from this pathway (Zadra et al., 2014). These results are consistent with previous studies investigating AMPK activation using a variety of prostate cancer cell lines, where multiple AMPK activators increased phosphorylation of AMPK, ACC, raptor, and inhibited phosphorylation of S6K (Zadra et al., 2014). Furthermore, our results demonstrating that Zyflamend increased fatty acid oxidation are consistent with AMPK activation as determined by radioactive CO_2 production using *in vivo* and *in vitro* models of prostate cancer (Zadra et al., 2014). Collectively, these results suggest that treatment of CWR22Rv1 cells with Zyflamend results in activation of AMPK that leads to a metabolic adaptation by which the cells shift from an anabolic state towards a more catabolic state, thereby diminishing fatty acid synthesis and other anabolic processes required to drive proliferation.

Replication of AMPK activation was demonstrated via the inhibition of mTORC1 signalling, and our results are similar to those of others who used a variety of tools to activate AMPK (Xiang et al., 2004; Zadra et al., 2014). Knockdown and overexpression experiments and the use of AICAR and compound C confirmed Zyflamend's role in regulating AMPK activation and its downstream signalling.

Four kinases have been identified that phosphorylate AMPKα at Thr172, liver kinase B1 (LKB1) (Hawley et al., 1996), calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) (Hawley et al., 2005), transforming growth factor-β activated protein kinase-1 (TAK1) (Herrero-Martin et al., 2009; Momcilovic, Hong, & Carlson, 2006), and most recently mixed-lineage kinase 3 (MLK3) (Luo, Jiang, Huang, Lu, & Luo, 2015). LKB1 is considered a tumour suppressor because mutation/inactivation of its gene is associated with a variety of cancers (Gan & Li, 2014). Expression of LKB1 has been reported to be lower in human prostate cancer tissue as compared to normal tissue (Xu, Cai, Liu, & Guo, 2014). Similar results were observed in PC3 and DU145 prostate cancer cell lines compared to control cells

(RWPE-1), and knockdown of LKB1 promoted their proliferation (Xu et al., 2014). Unlike LKB1, the activity of CaMKKβ is not sensitive to ATP : AMP ratios, but rather to increases in intracellular Ca^{2+} levels. CaMKKβ has been negatively associated with castration-resistant prostate cancer where loss of CaMKKβ increases proliferation and overexpression reduces tumour growth *in vivo* (Shima et al., 2012). There is also some evidence suggesting that deletion of MAP3K7, the gene that encodes for TAK1 protein, is positively associated with prostate cancer (Kluth et al., 2013), with a potential interaction between LKB1 and TAK1 (Xie et al., 2006). While we have not investigated upstream signalling of AMPK, our preliminary data show that chemical inhibitors of LKB1 (radicicol) and CaMKKβ (STO-609) reduced phosphorylation of AMPK (data not shown). Treatment with Zyflamend only in the presence of the CaMKKβ inhibitor restored pAMPK to untreated control levels. These data suggest that Zyflamend-stimulated phosphorylation of AMPK is independent of CaMKKβ activity and may be related in part to LKB1. The role of Zyflamend on the upstream regulation of AMPK will be further explored in future studies.

Recent data have strongly suggested that AMPK activation may also be linked to inhibition of PSA and androgen receptor expression, along with increases in p21 (Zadra et al., 2014), characteristics shared by Zyflamend treatment (Huang, Chen, et al., 2011; Huang, McEntee, et al. 2011; Huang et al., 2014). Castration-resistant forms of the disease coordinately involve activation of the androgen receptor in the absence of androgens coupled with higher expression of lipogenic genes compared with prostate cancer cells that are androgen-dependent (Ettinger et al., 2004; Swinnen, Ulrix, Heyns, & Verhoeven, 1997; Zadra et al., 2014).

5. Conclusion

In summary, Zyflamend is a polyherbal mixture whose components have been shown to act synergistically against cancer in a variety of models. It has been clearly established that activation of AMPK can inhibit mTORC1 signalling, the expressions of androgen receptor, p21, SREBP-1c and FASN, inhibit ACC activity, and increase fatty acid oxidation. Zyflamend also replicates these same effects. Based on these results and those previously published in other cancer models, investigations involving the effects of combinations of natural products, such as those found in Zyflamend, should be encouraged.

Conflict of interest

The following authors have no conflicts of interest: J. Whelan, D. Donohoe, E.-C. Huang and Y. Zhao. Six years ago New Chapter, Inc provided a grant (JW) to explore the impact of Zyflamend on castration-resistant prostate cancer; this support ended 4 years ago. New Chapter and its representatives were not informed of the current research nor consulted; have not been associated with the study design; in the collection, analysis, or interpretation of data; in the writing or review of the report; or in the decision to submit the paper for publication; nor have they provided any financial or non-financial support for this research. None of the authors have ever been provided honorariums or consulting fees by New Chapter. The research in the study was supported, in part, by a Hatch grant (USDA) through The University of Tennessee Research Experiment Station (JW). Neither The University of Tennessee Research Experiment Station nor anyone affiliated with them has been associated with the study design; in the collection, analysis, or interpretation of data; in the writing or review of the report; or in the decision to submit the paper for publication. This research has been reviewed and accepted by all authors. It has not been published or submitted for publication elsewhere.

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References

Bemis, D. L., Capodice, J. L., Anastasiadis, A. G., Katz, A. E., & Buttyan, R. (2005). Zyflamend, a unique herbal preparation with nonselective COX inhibitory activity, induces apoptosis of prostate cancer cells that lack COX-2 expression. *Nutrition and Cancer*, 52, 202–212. doi:10.1207/s15327914nc5202_10.

Braga, M. E., Leal, P. F., Carvalho, J. E., & Meireles, M. A. (2003). Comparison of yield, composition, and antioxidant activity of turmeric (Curcuma longa L.) extracts obtained using various techniques. *Journal of Agricultural and Food Chemistry*, 51, 6604–6611. doi:10.1021/jf0345550.

Burke, S. J., Karlstad, M. D., Conley, C. P., Reel, D., Whelan, J., & Jason, C. J. (2015). Dietary polyherbal supplementation decreases CD3(+) cell infiltration into pancreatic islets and prevents hyperglycemia in nonobese diabetic mice. *Nutrition Research*, 35, 328–336. doi:10.1016/j.nutres.2014.12.003.

Capodice, J. L., Gorroochurn, P., Cammack, A. S., Eric, G., McKiernan, J. M., Benson, M. C., Stone, B. A., & Katz, A. E. (2009). Zyflamend in men with high-grade prostatic intraepithelial neoplasia: Results of a phase I clinical trial. *Journal for the Society of Integrative Oncology*, 7, 43–51. doi:10.2310/7200.2009.0012.

Cheng, A. L., Hsu, C. H., Lin, J. K., Hsu, M. M., Ho, Y. F., Shen, T. S., Ko, J. Y., Lin, J. T., Lin, B. R., Ming-Shiang, W., Yu, H. S., Jee, S. H., Chen, G. S., Chen, T. M., Chen, C. A., Lai, M. K., Pu, Y. S., Pan, M. H., Wang, Y. J., Tsai, C. C., & Hsieh, C. Y. (2001). Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Research*, 21, 2895–2900.

Ekmekcioglu, S., Chattopadhyay, C., Akar, U., Gabisi, A., Jr., Newman, R. A., & Grimm, E. A. (2011). Zyflamend mediates therapeutic induction of autophagy to apoptosis in melanoma cells. *Nutrition and Cancer*, 63, 940–949. doi:10.1080/01635581.2011.586488.

Ettinger, S. L., Sobel, R., Whitmore, T. G., Akbari, M., Bradley, D. R., Gleave, M. E., & Nelson, C. C. (2004). Dysregulation of sterol response element-binding proteins and downstream effectors in prostate cancer during progression to androgen independence. *Cancer Research*, 64, 2212–2221. doi:10.1158/0008-5472.CAN-2148-2.

Flavin, R., Zadra, G., & Loda, M. (2011). Metabolic alterations and targeted therapies in prostate cancer. *Journal of Pathology*, 223, 283–294. doi:10.1002/path.2809.

Gan, R. Y., & Li, H. B. (2014). Recent progress on liver kinase B1 (LKB1): Expression, regulation, downstream signaling and cancer suppressive function. *International Journal of Molecular Sciences*, *15*, 16698–16718. doi:10.3390/ijms150916698; ijms150916698 [pii].

Hardie, D. G., Ross, F. A., & Hawley, S. A. (2012a). AMP-activated protein kinase: A target for drugs both ancient and modern. *Chemistry and Biology*, *19*, 1222–1236. doi:10.1016/j.chembiol.2012.08.019.

Hardie, D. G., Ross, F. A., & Hawley, S. A. (2012b). AMPK: A nutrient and energy sensor that maintains energy homeostasis. *Nature Reviews. Molecular Cell Biology*, *13*, 251–262. doi:10.1038/nrm3311.

Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D., & Hardie, D. G. (1996). Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *Journal of Biological Chemistry*, *271*, 27879–27887.

Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B.G., & Hardie, D.G. (2005). Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metabolism*, *2*, 9–19. doi:10.1016/j.cmet.2005.05.009; S1550-4131(05)00166-X [pii].

Herrero-Martin, G., Hoyer-Hansen, M., Garcia-Garcia, C., Fumarola, C., Farkas, T., Lopez-Rivas, A., & Jäättelä, M. (2009). TAK1 activates AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells. *EMBO Journal*, *28*, 677–685.

Howells, L. M., Moiseeva, E. P., Neal, C. P., Foreman, B. E., Andreadi, C. K., Sun, Y. Y., Hudson, E. A., & Manson, M. M. (2007). Predicting the physiological relevance of in vitro cancer preventive activities of phytochemicals. *Acta Pharmacologica Sinica*, *28*, 1274–1304. doi:10.1111/j.1745-7254.2007.00690.x.

Huang, E.-C., Chen, G., Baek, S. J., McEntee, M. F., Collier, J. J., Minkin, S., Biggerstaff, J. P., & Whelan, J. (2011). Zyflamend reduces the expression of androgen receptor in a model of castrate-resistant prostate cancer. *Nutrition and Cancer*, *63*, 1–10. doi:10.1080/01635581.2011.606956.

Huang, E.-C., McEntee, M. F., & Whelan, J. (2011). Zyflamend, a combination of herbal extracts, attenuates tumor growth in murine xenograph models of prostate cancer. *Nutrition and Cancer*, *64*, 749–760. doi:10.1080/01635581.2012.689413.

Huang, E.-C., Zhao, Y. C. G., Baek, S. J., McEntee, M. F., Minkin, S., Biggerstaff, J. P., & Whelan, J. (2014). Zyflamend, a polyherbal mixture, down regulates class I and class II histone deacetylases and increases p21 levels in castrate-resistant prostate cancer cells. *BMC Complementary and Alternative Medicine*, *14*, 68. doi:10.1186/1472-6882-14-68.

Kim, D. H., Sarbassov, D. D., Ali, S. M., Latek, R. R., Guntur, K. V., Erdjument-Bromage, H., Tempst, P., & Sabatini, D.M. (2003). GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Molecular Cell*, *11*, 895–904. doi:10.1016/S1097-2765(03)00114-X.

Kim, J. H., Park, B., Gupta, S. C., Kannappan, R., Sung, B., & Aggarwal, B. B. (2012). Zyflamend sensitizes tumor cells to TRAIL-induced apoptosis through up-regulation of death receptors and down-regulation of survival proteins: Role of ROS-dependent CCAAT/enhancer-binding protein-homologous protein pathway. *Antioxidants and Redox Signaling*, *16*, 413–427. doi:10.1089/ars.2011.3982.

Kluth, M., Hesse, J., Heinl, A., Krohn, A., Steurer, S., Sirma, H., Simon, R., Mayer, P. S., Schumacher, U., Grupp, K., Izbicki, J. R., Pantel, K., Dikomey, E., Korbel, J. O., Plass, C., Sauter, G., Schlomm, T., & Minner, S. (2013). Genomic deletion of MAP3K7 at 6q12-22 is associated with early PSA recurrence in prostate cancer and absence of TMPRSS2:ERG fusions. *Modern Pathology*, *26*, 975–983. doi:10.1038/modpathol.2012.236; modpathol2012236 [pii].

Kunnumakkara, A. B., Sung, B., Ravindran, J., Diagaradjane, P., Deorukhkar, A., Dey, S., Koca, C., Tong, Z., Gelovani, J. G., Guha, S., Krishnan, S., & Aggarwal, B. B. (2012). Zyflamend suppresses growth and sensitizes human pancreatic tumors to gemcitabine in an orthotopic mouse model through modulation of multiple targets. *International Journal of Cancer*, *131*, E292–E303. doi:10.1002/ijc.26442.

Luo, L., Jiang, S., Huang, D., Lu, N., & Luo, Z. (2015). MLK3 Phophorylates AMPK Independently of LKB1. *PLoS ONE*, *10*, e0123927. doi:10.1371/journal.pone.0123927; PONE-D-14–55927 [pii].

McEntee, M. F., Ziegler, C., Reel, D., Tomer, K., Shoieb, A., Ray, M., Neilsen, N., Lih, F. B., O'Rourke, D., & Whelan, J. (2008). Dietary n-3 polyunsaturated fatty acids enhance hormone ablation therapy in androgen-dependent prostate cancer. *American Journal of Pathology*, *173*, 229–241. doi:10.2353/ajpath.2008.070989.

Mohebati, A., Guttenplan, J. B., Kochhar, A., Zhao, Z. L., Kosinska, W., Subbaramaiah, K., & Dannenberg, A.J. (2012). Carnosol, a constituent of Zyflamend, inhibits aryl hydrocarbon receptor-mediated activation of CYP1A1 and CYP1B1 transcription and mutagenesis. *Cancer Prevention Research*, *5*, 593–602. doi:10.1158/1940-6207.CAPR-12-0002.

Momcilovic, M., Hong, S. P., & Carlson, M. (2006). Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. *Journal of Biological Chemistry*, *281*, 25336–25343. doi:10.1074/jbc.M604399200; M604399200 [pii].

Nagabhushan, M., Miller, C. M., Pretlow, T. P., Giaconia, J. M., Edgehouse, N. L., Schwartz, S., Kung, H. J., de Vere White, R. W., Gumerlock, P. H., Resnick, M. I., Amini, S. B., & Pretlow, T. G. (1996). CWR22: The first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both *in vivo* and in soft agar. *Cancer Research*, *56*, 3042–3046.

Pretlow, T. G., Wolman, S. R., Micale, M. A., Pelley, R. J., Kursh, E. D., Resnick, M. I., Bodner, D. R., Jacobberger, J. W., Delmoro, C. M., Giaconia, J. M., & Pretlow, T. P. (1993). Xenografts of primary human prostatic carcinoma. *Journal National Cancer Institute*, *85*, 394–398. doi:10.1093/jnci/85.5.394.

Rafailov, S., Cammack, S., Stone, B. A., & Katz, A. E. (2007). The role of Zyflamend, an herbal anti-inflammatory, as a potential chemopreventive agent against prostate cancer: A case report. *Integrative Cancer Therapy*, *6*, 74–76. doi:10.1177/1534735406298843.

Sandur, S. K., Ahn, K. S., Ichikawa, H., Sethi, G., Shishodia, S., Newman, R. A., & Aggarwal, B. B. (2007). Zyflamend, a polyherbal preparation, inhibits invasion, suppresses osteoclastogenesis, and potentiates apoptosis through down-regulation of NF-kappa B activation and NF-kappa B-regulated gene products. *Nutrition and Cancer*, *57*, 78–87. doi:10.1080/01635580701268295.

Shaw, R. J. (2009). LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. *Acta Physiologica*, *196*, 65–80. doi:10.1111/j.1748-1716.2009.01972.x.

Shima, T., Mizokami, A., Miyagi, T., Kawai, K., Izumi, K., Kumaki, M., Ofude, M., Zhang, J., Keller, E. T., & Namiki, M. (2012). Down-regulation of calcium/calmodulin-dependent protein kinase kinase 2 by androgen deprivation induces castration-resistant prostate cancer. *The Prostate*, *72*, 1789–1801. doi:10.1002/pros.22533.

Srinivasan, K. (2014). Antioxidant potential of spices and their active constituents. *Critical Reviews in Food Science and Nutrition*, *54*, 352–372. doi:10.1080/10408398.2011.585525.

Swinnen, J. V., Ulrix, W., Heyns, W., & Verhoeven, G. (1997). Coordinate regulation of lipogenic gene expression by

androgens: Evidence for a cascade mechanism involving sterol regulatory element binding proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 12975–12980.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernandez-Lopez, J., & Perez-Alvarez, J. A. (2011). Spices as functional foods. *Critical Reviews in Food Science and Nutrition*, 51, 13–28. doi:10.1080/10408390903044271; 932225231 [pii].

Wright, J. L., & Stanford, J. L. (2009). Metformin use and prostate cancer in Caucasian men: Results from a population-based case-control study. *Cancer Causes and Control*, 20, 1617–1622. doi:10.1007/s10552-009-9407-y.

Xiang, X., Saha, A. K., Wen, R., Ruderman, N. B., & Luo, Z. (2004). AMP-activated protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms. *Biochemical and Biophysical Research Communications*, 321, 161–167. doi:10.1016/j.bbrc.2004.06.133.

Xie, M., Zhang, D., Dyck, J. R., Li, Y., Zhang, H., Morishima, M., Mann, D. L., Taffet, G. E., Baldini, A., Khoury, D. S., & Schneider, M. D. (2006). A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 17378–17383. doi:10.1073/pnas.0604708103; 0604708103 [pii].

Xu, P., Cai, F., Liu, X., & Guo, L. (2014). LKB1 suppresses proliferation and invasion of prostate cancer through hedgehog signaling pathway. *International Journal of Clinical and Experimental Pathology*, 7, 8480–8488.

Yan, J., Xie, B., Capodice, J. L., & Katz, A. E. (2012). Zyflamend inhibits the expression and function of androgen receptor and acts synergistically with bicalutimide to inhibit prostate cancer cell growth. *The Prostate*, 72, 244–252. doi:10.1002/pros.21426.

Yang, P., Cartwright, C., Chan, D., Vijjeswarapu, M., Ding, J., & Newman, R. A. (2007). Zyflamend-mediated inhibition of human prostate cancer PC3 cell proliferation: Effects on 12-LOX and Rb protein phosphorylation. *Cancer Biology and Therapy*, 6, 228–236. doi:10.4161/cbt.6.2.3624.

Yang, P., Sun, Z., Chan, D., Cartwright, C. A., Vijjeswarapu, M., Ding, J., Chen, X., & Newman, R. A. (2008). Zyflamend reduces LTB4 formation and prevents oral carcinogenesis in a 7,12-dimethylbenz[alpha]anthracene (DMBA)-induced hamster cheek pouch model. *Carcinogenesis*, 29, 2182–2189. doi:10.1093/carcin/bgn181.

Zadra, G., Photopoulos, C., Tyekucheva, S., Heidari, P., Weng, Q. P., Fedele, G., Liu, H., Scaglia, N., Priolo, C., Sicinska, E., Mahmood, U., Signoretti, S., Birnberg, N., & Loda, M. (2014). A novel direct activator of AMPK inhibits prostate cancer growth by blocking lipogenesis. *EMBO Molecular Medicine*, 6, 518–538. doi:10.1002/emmm.201302734.

Zakikhani, M., Dowling, R. J., Sonenberg, N., & Pollak, M. N. (2008). The effects of adiponectin and metformin on prostate and colon neoplasia involve activation of AMP-activated protein kinase. *Cancer Prevention Research*, 1, 369–375. doi:10.1158/1940-6207.CAPR-08-0081.

Zhao, Y., Collier, J. J., Huang, E.-C., & Whelan, J. (2014). Turmeric and Chinese goldthread synergistically inhibit prostate cancer cell proliferation and NF-kB signaling. *Functional Foods in Health and Disease*, 4, 312–339.