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Characterizing the function of extracellular protein kinase A in angiogenesis and the effects of Zfp68 and pharmacological inhibitors in adipogenesis

by

Maria Szkudlarek-Mikho

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Science

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An Abstract of

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Cyclic AMP (cAMP) is a ubiquitous intracellular second messenger whose major intracellular function in eukaryotes is the activation of protein kinase A (PKA). PKA, a well known member of the serine-threonine protein kinase superfamily, is implicated in the control of a variety of cellular processes. While the role of intracellular PKA holoenzyme in cell growth has been widely investigated, little is known about the presence of PKA activity in the extracellular surface of cells. Here we show, using the *in utero* chicken embryo chorioallantoic membrane assay, that the extracellular form of PKA (ECPKA), from conditioned media of cultured cancer cells, inhibits angiogenesis. Whether the aberrant release of ECPKA is just a bystander effect or might have an important function in cancer development warrant further investigation and so does the potential application as a biomarker in cancer.

Obesity, a multifactorial and chronic disorder has risen to alarming levels. The World Health Organization has estimated that worldwide over one billion adults are overweight, including at least 300 million of them being obese. Obese patients are at a higher risk of hypertension and increased risk of cardiovascular disease, dyslipidemia, osteoarthritis, and diabetes. Obesity treatment has become a major public health concern and burden on the health care system. Current pharmacotherapeutic options for treating obesity and related metabolic disorders remain very limited and ineffective. The development of more effective drugs has become a priority. Here we present two agents with anti-adipogenic activity: prieurianin and salinomycin.

Prieurianin, a limonoid, causes weight loss by reducing energy intake in morbidly obese mice and in mice on high-calorie diet. Prieurianin is also anti-adipogenic by inhibiting the proliferation and differentiation of preadipocytes into adipocytes, and induces either dedifferentiation or delipidation of mature adipocytes. Microarray analysis revealed number of gene expression changes following treatment with prieurianin, including a number of genes involved in fat metabolism. However, we focused our attention in the subset of early response genes, among which, we found previously identified transcriptional repressor zinc finger protein 68 (Zfp68).

Salinomycin is a coccidiostat and antibacterial agent that belongs to a family of a polyether ionophore antibiotics. Here, we show that salinomycin inhibits the differentiation of preadipocytes into adipocytes and is a potent inhibitor of transcriptional activity of major adipogenic transcription factors. These results suggest that ionophore antibiotics or their derivatives could be further exploited as novel anti-obesity therapies and as pharmacological probes for the study of adipose biology.

Dedicated to Jadwiga and Boguslaw Szkudlarek

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Chapter I. Introduction and Literature Review

Cyclic AMP and the cAMP – dependent protein kinase (PKA)

The protein kinases constitute one of the largest gene families, encoded by 2% of human genome, and about 4% of plant genomes (Tchieu, et al., 2003). The protein kinases modify approximately a third of all human proteins and they are an essential part of the machinery that is activated in response to stress; are necessary for memory, and directly involved in orchestrating cell death. Because of their extensive participation in many cellular events, the protein kinases are associated with different diseases. Consequently, these enzymes have rapidly emerged as major therapeutic targets.

Within the large and very diverse family of protein kinases, cAMP-dependent protein kinase (PKA) is one of the simplest and best understood (Beebe, Redmon, Blackmore, & Corbin, 1985; Francis & Corbin, 1994; Taylor, Buechler, & Yonemoto, 1990). The signal transduction pathway of cyclic AMP is generally present in all eukaryotic cells and is essential to such processes as regulation of metabolism (Krebs & Beavo, 1979), cell growth and differentiation (Cho-Chung, 1990), and gene expression (Lalli & Sassone-Corsi, 1994). PKA was one of the first to be discovered

in 1968 (Walsh, Perkins, & Krebs, 1968), its kinetic properties have been well-defined and the elucidation of its structure provided the first three-dimensional prototype for this large and highly diverse enzyme family. The simplicity originates from the fact that PKA is composed of two types of two genetically distinct subunits: two catalytic (C) and two regulatory (R) subunits (Taylor, et al., 2008), which form a tetramer R_2C_2 (Figure 1). The regulatory subunits of PKA maintain the catalytic subunit in the inactive state and couples cAMP binding to kinase activation (Taylor, et al., 1990). In addition, the regulatory subunit localizes the kinase to specific intracellular locations via its interaction with A kinase-anchoring proteins (AKAPs) (Colledge & Scott, 1999). In the absence of cAMP, the two regulatory subunits keep two substrate binding (catalytic) subunits in an inactive state. Activation of PKA occurs when the concentration of cAMP rises in response to specific cellular stimuli, then four molecules of cAMP bind to the regulatory subunits, two to each subunit. When cAMP binding sites are occupied, this causes the R subunit to change confirmation and the holoenzyme dissociates into an $R_2(cAMP)_4$ dimer and two free catalytically active C kinase subunits. Now the activated C subunits can phosphorylate a number of target proteins at serine and threonine residues, both in the cytoplasmic and in the nuclear compartments (Chin, et al., 2002).

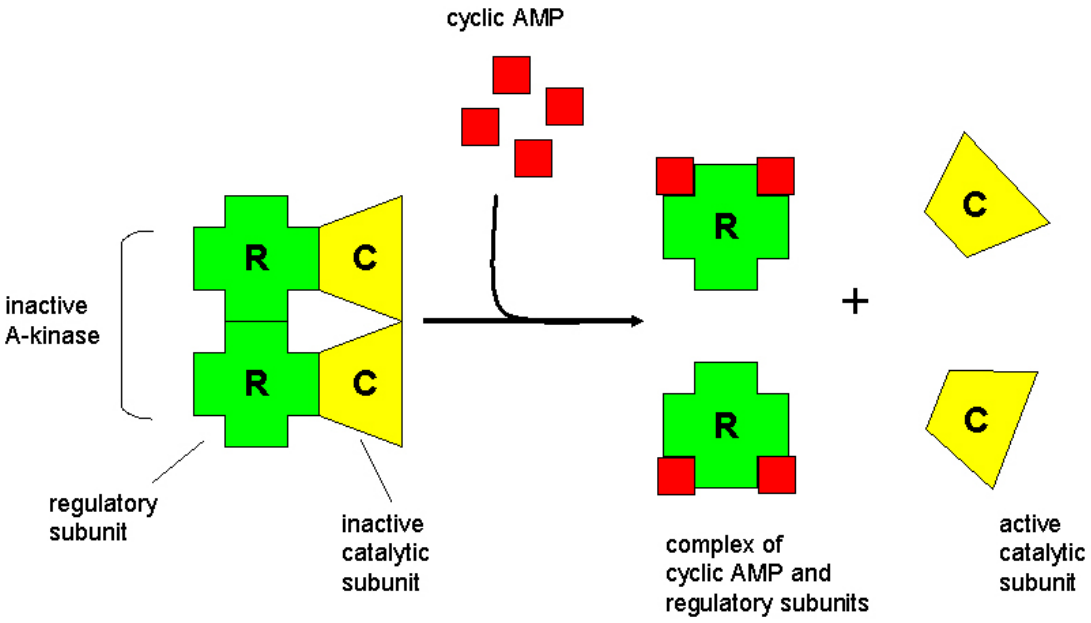

Fig.1. Cyclic AMP-dependent protein kinase (PKA) is a holoenzyme consisting of two regulatory (R) subunits and two catalytic (C) subunits. Activation of PKA occurs when cAMP binds to the R subunit dimmer causing the catalytic subunit to be unleashed so that it can phosphorylate its protein substrates (modified from Molecular Biology of the Cell).

There are three catalytic C subunits isoforms (Cα, Cβ and Cγ) and two families of regulatory R subunits (RI and RII) with distinct cAMP binding properties. The two major R isoforms have been further distinguished as RIα, RIβ, RIIα, and RIIβ. The isoforms are functionally nonredundant, and isoform diversity is a major mechanism for achieving specificity in PKA signaling (Amieux & McKnight, 2002). Based on the R subunit composition, there are two major types of PKA in mammalian cells, Type I and Type II. These isozymes can compose from either homo- or heterodimers of the R subunits yielding holoenzyme complexes of PKA with a

number of combinatorial configurations, including $RI\alpha_2C_2$, $RI\beta_2C_2$, $RII\alpha_2C_2$, $RII\beta_2C_2$, and $RI\alpha RI\beta C_2$ (Tasken, et al., 1993). Significantly, the ratios of PKA-I to PKA-II differ dramatically during cell development, differentiation and transformation (Lohmann & Walter, 1984). Furthermore the presence of multiple C subunit genes adds to the diversity and complexity of the various holoenzyme complexes that may be found in cells (Skalhegg & Tasken, 2000). In addition to diverse biochemical and functional properties, these genetically distinct isozymes have also been shown to have different patterns of expression and localization (Pawson & Scott, 1997) thus conferring the broad specificity that PKA may have on a large number of physiological processes and signaling mechanisms in cells in response to the differential effects of cAMP.

The biochemical mechanisms and effects of cAMP and PKA occur primarily inside the cell and PKA is known to be an intracellular enzyme (Burns-Hamuro, et al., 2003). However, there are also reports about its extracellular form. An extracellular form was first reported in the rat C-6 glioma cells in which PKA complex was found to be associated with the outer cell surface (Schlaeger & Kohler, 1976). Further, the presence of extracellular PKA was also observed in rat adipocytes, spermatozoa and human platelets, and pulmonary microvascular endothelial cells (Elalamy, Said, Singer, Couetil, & Hatmi, 2000; Kang, Gates, Chiang, & Kang, 1979; Majumder, 1978). The term *ectoenzyme* was created for membrane-bound enzyme whose catalytic activity is localized on the outer surface of cell membrane, whereas *exoenzyme* or extracellular enzyme, is an enzyme that is secreted by a cell outside of that cell, and can be isolated as soluble protein (Ehrlich, 1996). The above explanations imply that PKA can be localized outside of cell functioning as ecto-PKA once associated with the outer membrane or as exo-PKA in a free soluble form.

It was shown that the free C subunit of PKA (PKAc) was detected in the conditioned media of various cultured cancer cells (Cho, Lee, & Cho-Chung, 2000; Cvijic, Kita, Shih, DiPaola, & Chin, 2000).

Biochemical and immunological characterization revealed that this extracellular PKA is a type I PKA. Knowing the enhanced sensitivity to cAMP activation of type I vs. type II PKA holoenzyme (Planas, Cummings, Idzerda, & McKnight, 1999), this ECPKA expression may be an important mechanism for regulation of the cellular PKA type I/ type II holoenzyme ratio, and for maintaining regulation of the C subunit levels in the cell.

The activity of ecto-PKA was observed by phosphorylation of the PKA specific substrate – Kemptide, occurring presumably at the surface of HeLA cells (Kubler, et al., 1989). Moreover, the presence of PKA has been observed on the external surface of human colon carcinoma LS-174T cells (Kondrashin, Nesterova, & Cho-Chung, 1999). Furthermore, the free C subunit of PKA (PKAc) was found to be released and detected in the serum samples of a large number of cancer patients, compared to a low level of normal controls (Cho, Lee, et al., 2000; Cho, Park, et al., 2000; Cvijic, et al., 2000). The release of ECPKA in peripheral blood was further confirmed by additional findings (Kita, et al., 2004; H. Wang, et al., 2007). This PKA designated as extracellular protein kinase A (ECPKA), is distinctive from the intracellular tetrameric holoenzyme.

The PKA kinase activity is specifically inhibited by the protein kinase A peptide inhibitor (PKI), but not by the PKC-specific peptide inhibitor. The secreted ECPKA is constitutively active at various levels, in serum free media from a variety of tissue culture cell lines. Since the activity of the ECPKA does not require

activation by cAMP, it is possible that only the C subunit is present extracellulary, independent of the PKA holoenzyme (Cvijic, et al., 2000).

The presence of PKA activity outside the cell is interesting but not very well understood. The unusual localization of these forms of PKA holoenzyme in the extracellular milieu raises the question of cell lysis. However, that is not the case since other intracellular protein markers, such as mitochondrial lactate dehydrogenase, were not detected either in the serum or in the conditioned media of cultured cancer cells (Cvijic, et al., 2000)

More recently, autoantibody against ECPKA has been detected in the serum of a large number of patients with various types of cancer by an autoantibody enzyme immunoassay (Nesterova, et al., 2006), thus suggests that ECPKA autoantibody may serve as a universal biomarker for cancer detection.

Activation of intracellular PKA by cAMP and the subsequent protein phosphorylation mediated by the kinase in the regulation of cell growth, development, metabolism, and gene expression (Beebe, 1994; Chin, et al., 2002) have been widely investigated for more than four decades. Hence, the discovery of free PKAc in peripheral blood in cancer is intriguing. Though the function of the secreted kinase is currently unknown, it is clear that there are marked differences between ECPKA and intracellular PKA. Therefore, it is imperative to elucidate the physiological role of ECPKA, as well as identifying its extracellular targets of phosphorylation in cancer. We investigated the role of ECPKA in angiogenesis and characterized the PKAc isozymes released by cancer cells.

Angiogenesis

Angiogenesis, the growth of new capillary blood vessels in the body from preexisting vasculature, is an important natural process occurring, both in the normal and diseased state. The vascular endothelial cell (EC) is the major player in angiogenesis, but other cells like fibroblasts, immune cells, pericytes and inflammatory cells contribute to its regulation. Fetal development requires constant activation of angiogenesis, but after birth, the vascular system is completely formed and angiogenesis is activated mostly during tissue repair and during the female reproductive cycle (Ferrara, et al., 1996). The development of a new blood vessel requires at least four steps: degradation of the basal lamina that covers existing blood vessels, migration toward an angiogenic signal, proliferation, and formation of new tubular structures. In the normal vasculature, this process is very complex and involves a tight regulation between pro- and anti-angiogenic factors. Insufficient or excessive angiogenesis is related to several diseases. Coronary artery disease, stroke, and delayed wound healing are examples of diseases where lack of angiogenic factors cause insufficient blood vessel growth, and circulation is not properly restored. On the contrary, progression of numerous pathologies like cancer, age-related macular degeneration (AMD) and atherosclerosis require activation of angiogenesis (Pandya, Dhalla, & Santani, 2006).

The observation that angiogenesis occurs around tumors was made nearly 100 years ago (Goldmann, 1908). In 1971, Folkman proposed that tumor growth and metastasis are angiogenesis dependent, and therefore blocking angiogenesis could be a strategy to arrest tumor growth (Folkman, 1971). Further, it was shown that cells in

pre-cancerous tissue acquire angiogenic capacity on their way to becoming cancerous (Gullino, 1978). It is well known that angiogenesis and the production of angiogenic factors play important roles in tumor growth, invasion, and metastasis (Ribatti, Vacca, & Dammacco, 1999). In fact, an increasing number of experimental evidence indicates that tumor growth and lethality are both dependent on angiogenesis. As tumor cells proliferate, the center of the tumor is undersupplied in oxygen and nutrients. In order to promote tumor growth, and to stimulate angiogenesis, tumor cells secrete numerous pro-angiogenic factors, including b-FGF (New & Yeoman, 1992) and VEGF (Folkman & Shing, 1992).

The classical assays for studying angiogenesis *in vivo* include the hamster cheek pouch, rabbit ear chamber, dorsal skin and air sac, the chick embryo chorioallantoic membrane (CAM) and iris and corneal vascularization of rodent eye. One of the most commonly used methods to study angiogenic activity is the chick chorioallantoic membrane (CAM). It has been used to identify almost all of the known angiogenic factors (Ribatti, Nico, Vacca, & Presta, 2006). The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane mediating nutrient and gas exchanges until hatching. Since it has a dense capillary network, it has been commonly used in vivo to study both angiogenesis and anti-angiogenesis in response to tissues, cells or soluble factors (Ribatti, 2008).

We investigated the role of ECPKA and characterized the PKAc isozymes released by cancer cells by utilizing the chick embryo chorioallantoic membrane (CAM) assay.

Obesity

During the past two decades, obesity has become a leading public health problem worldwide. Obesity is a condition in which the natural energy reserve stored in fatty tissue exceeds healthy limits. For humans, it is commonly defined as a body mass index (BMI) of 30 kg/m^2 or higher. The prevalence of obesity is rapidly on the rise not only in industrialized nations, but also in developed countries (Speakman, 2004). In the United States of America, almost 65% of the population is overweight and nearly 20 % are obese (Speakman, 2004). Total health-care costs accounted to obesity and overweight will reach around 900 billion US dollars by 2030, accounting for 16–18% of total US health-care costs (Y. Wang, Beydoun, Liang, Caballero, & Kumanyika, 2008).

Obesity is a multifactorial, chronic disorder that has reached epidemic proportions in most industrialized countries and is threatening to become a global epidemic. The problem of overweight/obesity has been identified as one of the major cardiovascular disease (CVD) risk factors since 1998 (Eckel & Krauss, 1998). Obese individuals are at higher risk for coronary artery disease, hypertension, hyperlipidemia, diabetes mellitus, cancers, cerebrovascular accidents, and various other malaises (Calle, Rodriguez, Walker-Thurmond, & Thun, 2003; Li, Bowerman, & Heber, 2005). Although most obesity appears to be caused by energy imbalance, obesity is a complex condition, with environmental and genetic contributory factors. As a whole, overweight/obesity predisposes or is associated with numerous cardiac

complications such as coronary heart disease, heart failure, and sudden death through its impact on the cardiovascular system (Poirier, et al., 2006).

Pharmacotherapy of Obesity

The approach for obesity treatment generally aims at shifting the balance between energy consumption and energy expenditure (Dehghan, Akhtar-Danesh, & Merchant, 2005). The act of "dieting" undertaken by an individual or more rigorous diet restriction prescribed by a doctor represents the simplest method to combat obesity, and yet limiting caloric consumption is difficult for a variety of societal and lifestyle reasons. Treatment options for obesity epidemics are rather limited (Kirk, Scott, & Daniels, 2005). Currently in the US, there are only two effective pharmacological agents approved for long-term use by the Food and Drug Administration (FDA) for obesity. Orlistat blocks up to 30% of ingested fat in the gastrointestinal tract, whereas sibutramine inhibits the reuptake of neurotransmitters to promote satiety and prevent overconsumption and is only approved for patients over the age of 18. Adverse effects of these medications include gastrointestinal complications (orlistat), increases in blood pressure and heart rate (sibutramine) and high attrition rates for both drugs (Padwal & Majumdar, 2007). In fact, just recently, sibutramine has been withdrawn from the European market because of unacceptable side effects.

In cases of severe obesity, bariatric surgery for adults has been shown to be effective, and is gaining prominence for use in adolescents. Obese adolescents with a BMI of 40 or more and serious medical complications associated with their obesity are considered good candidates for the surgery (Kirk, et al., 2005). The costs and

risks of this procedure, and its limitation only in severe cases, restrain its applicability to the general population.

Along with the study of the enormity of cardiovascular and metabolic disease and epidemiological risk patterns, novel therapeutics are required (Rana, Nieuwdorp, Jukema, & Kastelein, 2007).

Adipogenesis

The primary function of adipose tissue is to store energy in the form of triglycerides during periods of energy excess and to release the energy during fasting or starvation as free fatty acids and glycerol. Adipose tissue is not only a structural component of the body and a place for energy storage, but also an important regulator of energy homeostasis through secretion of signal molecules such as leptin and adipsin (Rosen & Spiegelman, 2000). Excess adipose tissue can be a result of both hypertrophy (increased lipid accumulation) and hyperplasia (increased proliferation/differentiation) of adipocytes.

Adipogenesis is the process of preadipocyte differentiation into a mature fat cell. During adipogenesis, mesenchymal precursor cells differentiate first to adipocytes and mature into white or brown adipose tissues. While the major function of white adipose tissue (WAT) is storage, brown adipose tissue (BAT) dissipates energy as heat through the uncoupling of respiration from the synthesis of ATP, which is conferred by the increased number of mitochondria and expression of uncoupling protein-1 (Nedergaard *et al*., 1986). BAT may also function to protect against obesity. Depots of brown adipocytes are highly diminished in adult humans, but are more prominent in mature rodents (Nedergaard *et al*., 1986).

The mechanism controlling the process of preadipocyte proliferation and differentiation has been extensively studied *in vitro*. Several molecular events and transcriptional pathways that govern differentiation of preadipocytes to adipocytes have been identified. These discoveries were possible largely due to the establishment of the 3T3-L1 and 3T3-F442 preadipocytes cell lines from Swiss 3T3 cells developed by Howard Green in the 1970s (Green & Kehinde, 1975; Green & Meuth, 1974). The 3T3-L1 is one of the best-characterized cell culture systems for the study of adipogenesis as it recapitulates many of the features *in vivo*. Grown to confluence, under appropriate hormonal stimuli: methylisobutylxanthine (MIX), dexamethasone and insulin, preadipocytes can differentiate into cells that morphologically and ultrastructurally resemble mature adipocytes. Subsequently cellular and molecular analyses of these cells have shown that they posses the functional characteristics of fat cells. The effects of insulin on preadipocytes are not mediated by the insulin receptor, but instead occur through activation of the insulin-like growth factor 1 receptor (IGF-1 R). This cross-activation by insulin occurs with a super physiological dose of insulin (Rosen & Spiegelman, 2000). MIX is a phoshodiesterase inhibitor which increases levels of cAMP (Rosen, Walkey, Puigserver, & Spiegelman, 2000). Dexamethasone is a potent synthetic member of the glucocorticoid class of steroid hormones activating the glucocorticoid receptor (GR). The first stage of differentiation is growth arrest, which is achieved in culture by contact inhibition. Following growth arrest, the cells enter the second phase called clonal expansion in which the cell cycle goes one or two rounds upon treatment with the differentiation cocktail. After clonal expansion, cells enter final and permanent growth arrest and undergo terminal differentiation into mature adipocytes.

Accordingly, better understanding of the cellular and molecular mechanisms underlying adipose tissue development is necessary to identify novel molecular processes that may be novel targets for the development of anti-obesity drugs.

Transcriptional Control of Adipocytes Differentiation

The major transcription factors involved in adipogenesis include proteins belonging to the CCAAT/enhancer binding protein family and the peroxisome proliferator activated receptor γ (PPAR γ) (Figure 2). In the presence of hormonal signals that stimulate differentiation, there is rapid and transient induction of C/EBPβ and C/EBPδ (Darlington, Ross, & MacDougald, 1998). These factors in turn induced the expression of their respective target genes, which include C/EBPα and PPARγ, which are expressed right at the end of the clonal expansion stage. Increased expression of PPARγ, and C/EBPα will lead to expression of adipocyte specific genes that characterize the mature adipocyte. These genes are involved in insulin sensitivity, such as fatty acid synthase (FAS), lipoprotein lipase (LPL), acetyl CoA carboxylase and fatty acid binding protein (aP2). Also there are secreted products such as leptin, adipsin, and adiponectin (Rosen, 2005).

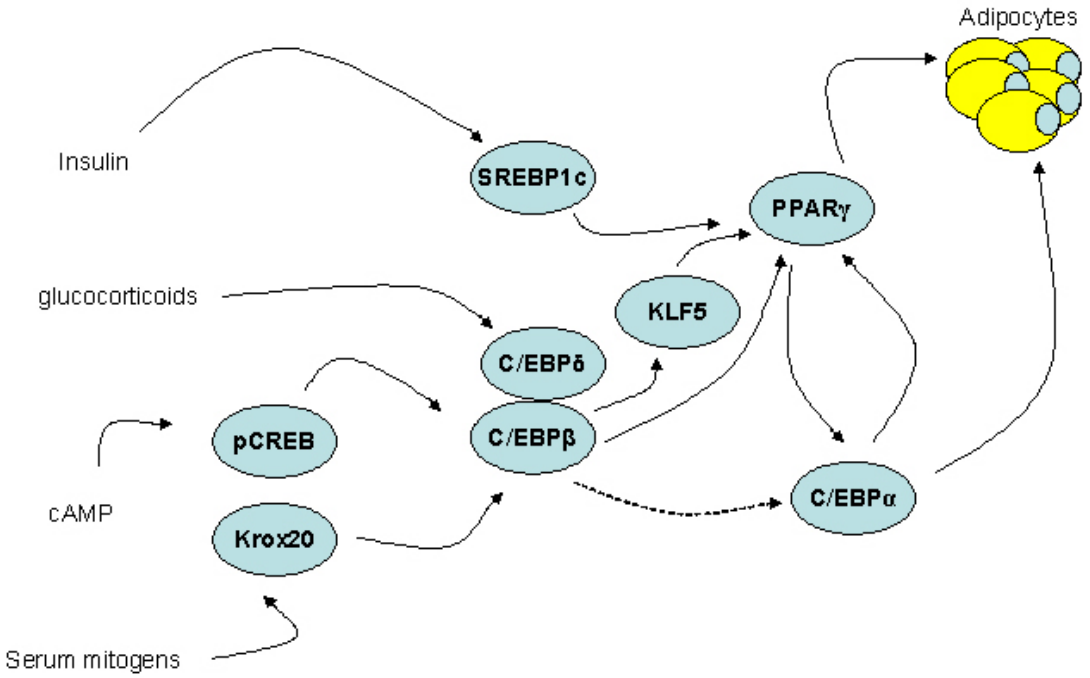

Figure 2. Induction of adipogenesis by a cascade of transcription factors. Exposure of preadipocytes to a cocktail of adipogenic inducers comprised of insulin, glucocorticoids, agents that increase cAMP level (isobutylmethylxanthine), and fetal bovine serum activates expression of several transcription factors. PPARγ then induces C/EBPα expression, and together, these factors oversee terminal adipogenesis (Farmer, 2006)

Zinc finger proteins

Zinc finger proteins are small protein domains in which zinc plays a structural role to stabilize the domain. This new class of proteins, discovered in 1985, can recognize and bind to a specific sequences of DNA, and regulate their activity (Miller, McLachlan, & Klug, 1985). Zinc finger proteins, which are extremely abundant in

higher eukaryotes, typically function as interaction modules and bind to nucleic acids, proteins and small molecules (Krishna, Majumdar, & Grishin, 2003). The most common type is the C_2H_2 type, or "classical" zinc finger (Wolfe, Nekludova, & Pabo, 2000). The primary role of C_2H_2 fingers is to bind to DNA segments and control transcription of target genes together with other factors (Iuchi, 2001). They are not only ubiquitous, but also the most frequent proteins within eukaryotic proteomes (Brayer & Segal, 2008). It is estimated that around 1% of total mammalian proteins are C_2H_2 zinc finger proteins, and to date, about one hundred thirty-three species of C_2H_2 type zinc finger cDNA have been identified in human brain alone (Klug, 1999).

Our work focused on a novel Krüppel-like zinc finger protein containing the evolutionarily conserved Krüppel-associated box domain, zinc finger protein (Zfp68). Krüppel-related zinc finger proteins, with around 500 members in the human genome, probably constitute the largest individual family of transcription factors in mammals. Approximately 30% of these proteins carry a potent repressor domain called the Krüppel associated box (KRAB). The KRAB domain, which is found in the amino-terminal region of the proteins, behaves as a transcriptional repressor domain by binding to corepressor proteins, whereas the C_2H_2 zinc-finger motifs bind DNA. The functions currently proposed for members of the KRAB-containing protein family include transcriptional repression of RNA polymerase I, II, and III promoters and binding and splicing of RNA. Zinc finger protein 68 belongs to a family of KRAB Zinc Finger Proteins. It has been shown that Zfp68 represses transcription and interact with KRAB-associated protein-1 (KAP-1) (Agata, Matsuda, & Shimizu, 1999). Therefore, chapter 2 will center on the characterization of Zfp68, a novel anti-adipogenic transcription factor.

Ionophore Antibiotics

The generic term ionophores was used for the first time in 1968 to describe all carboxylic polyethers that fit the classical definition of antibiotics (Watanabe, Watanabe, Kuramitsu, & Maruyama, 1981). Ionophores are lipid soluble molecules synthesized by microorganisms to transport ions across lipid bilayers of cell membranes. Since the mid-1970's antimicrobials are used extensively in livestock farming as growth promoters and to improve health and welfare of animals by controlling coccidiosis and decreasing the shedding of zoonotic pathogens (Butaye, Devriese, & Haesebrouck, 2003; Sapkota, Lefferts, McKenzie, & Walker, 2007). Ionophore antibiotics are used as growth promoters because they improve feed efficiency and increase the rate of weight gain. The effect on growth may be due to a combination of both fewer normal intestinal flora and a smaller number of harmful bacteria.

Most ionophore antibiotics are produced by *Streptomyces* spp. (Benno, Endo, Shiragami, & Mitsuoka, 1988). Examples of ionophores that either are used commercially or have been investigated for use in growing animals are monensin, lasalocid, tetronasin, salinomycin, lysocellin, narasin, nigericin, laidlomycin and valynomycin and they are the most successful anticoccidial agents. The United States Food and Drug Administration accepted the use of antibiotics as animal additives without veterinary prescription in 1951 (Jones & Ricke, 2003). Also in the 1950s and 1960s, each European state permitted its own national regulations about the use of antibiotics in animal feeds. However, due to the concerns of spreading resistance to

antibiotics used in human medical treatment, the European Commission has proposed prohibiting the use of antibiotics as growth promoters for animal feed by January 2006 (Castanon, 2007).

Unique structural features, including multiple cyclic ethers, a free carboxylic acid group at one end of the molecule, characterize the ionophore antibiotics and a terminal alcohol group at the other, such that they are described as polyether antibiotics. These ionophores readily form lipid-soluble cyclic complexes with polar cations, such as Li^{+}, Na^{+}, K^{+}, Cs^{+}, NH^{4+}, Ca^{2+}, Mg^{2+} and Cu^{2+} (Volmer & Lock, 1998).

Generally, ionophores have been found safe and effective in the target animals receiving recommended dosage levels (Novilla, 1992). However, the potential human health impacts associated with the use of these antimicrobials in animal feeds have not been fully examined. This widespread use of antibiotics administered at subtherapeutic levels in feed and water to promote growth and improve feed efficiency raises public health concern with the emergence of antibiotic-resistant pathogenic strains of bacteria. In light of the use of ionophore antibiotics in poultry and other animal farming practices, the question is raised about the potential impact of consuming meat tainted with growth-promoting antibiotics on human health, thus prompting us to ask whether these antibiotics may have biological effects on adipose tissues. It should be noticed, that the total production of antibiotics can be estimated between 100,000–200,000 tons annually and the human population is being influenced, directly or indirectly (from the environment) by this amount of antimicrobial agents. A twentieth-century increase in human height and the obesity of the population has been observed since the mass consumption of antibiotics 40–50 years ago. We hypothesize that there is an association between antibiotic

consumption and the increase of human growth and obesity. In chapter three, we tested the ionophore antibiotics in adipocytes differentiation assay and further focused the remaining of studies largely on the anti-adipogenic properties of a single agent, salinomycin.

Chapter II: Manuscript 1

Inhibition of Angiogenesis by Extracellular Protein Kinase A

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ABSTRACT

The cyclic-AMP dependent protein kinase (PKA) signaling pathway regulates cell growth, development, metabolism, and gene expression. Peripheral blood of cancer patients but not normal individuals, shows increased catalytic subunit levels of PKA (PKAc). We showed here that this extracellular form of PKAc (ECPKA) from conditioned media of cultured cancer cells as well as purified PKAc inhibit angiogenesis, using the *in utero* chicken embryo chorioallantoic membrane assay. Inhibition of angiogenesis is partially reversed by PKI, a peptide inhibitor of PKA, thus suggesting an anti-angiogenic role for ECPKA. The significance of ECPKA in cancer is discussed.

Keywords: ECPKA, angiogenesis, extracellular, PKA, chorioallantoic

INTRODUCTION

The cAMP-dependent protein kinase (PKA) is a ubiquitous tetrameric holoenzyme composed of two regulatory (R) and two catalytic (C) subunits, activatable by cAMP that dissociates into a dimer of R and two free C subunits (Matsuda, et al., 2001). The enzyme is intracellularly localized (Burns-Hamuro, et al., 2003). It was first reported in prostate and various other cancers that the C subunit kinase (PKAc) is released and found in the peripheral blood of cancer patients (Cho, Park, et al., 2000; Cvijic, et al., 2000). This anomalously secreted PKAc is constitutively active and does not require activation by cAMP, and its activity is inhibited specifically by the protein kinase A peptide inhibitor (PKI). It was further determined that PKAc is also found in the conditioned media of various cultured cancer cells (Cho, Lee, et al., 2000; Cvijic, et al., 2000). This extracellular form of PKAc is termed ECPKA, which is distinct from the intracellular tetrameric holoenzyme that is activated by cAMP.

The release of ECPKA into the extracellular milieu is not due to cell lysis, because intracellular marker protein, such as the mitochondrial lactate dehydrogenase, was neither detectable in the serum nor in the conditioned media of cultured cancer cells (Cvijic, et al., 2000). Moreover, the release of ECPKA in peripheral blood has been further confirmed in other reports (Kita, et al., 2004; H. Wang, et al., 2007). More recently, autoantibody against ECPKA has been detected in the serum of a large number of patients with various types of cancer by an autoantibody enzyme

immunoassay (Nesterova, et al., 2006), thus suggesting that ECPKA autoantibody may serve as a universal biomarker for cancer detection.

Activation of intracellular PKA by cAMP and the subsequent protein phosphorylation mediated by the kinase in the regulation of cell growth, development, metabolism, and gene expression (Beebe, 1994; Chin, et al., 2002) have been widely investigated for more than four decades. Hence, the discovery of free PKAc in peripheral blood in cancer is intriguing. Though the function of the secreted kinase is currently unknown, it is clear that there are marked differences between ECPKA and intracellular PKA. Therefore, it is imperative to elucidate the physiological role of ECPKA, as well as identifying its extracellular targets of phosphorylation in cancer. In this report, we investigated the role of ECPKA in angiogenesis and characterized the PKAc isozymes released by cancer cells. The significance of ECPKA in cancer is also discussed.

MATERIAL AND METHODS

Chicken Chorioallantoic Membrane (CAM) Assay

The chicken embryo chorioallantoic membrane (CAM) assay is conducted as describe before (Ausprunk, Knighton, & Folkman, 1974). Briefly, one day old fertilized eggs (Hertzfeld Poultry Farms, Waterville, OH, USA) were cleaned with Spor-Klenz solution (Steris, Mentor, OH, USA) and the chick embryos were incubated for 3 days at 37°C, in 0.5% CO_2 with 85% humidity. CAM was separated from the egg's shell by cracking the egg and dropping the chick embryo with intact yolks into 100 mm tissue culture dish containing 5 ml of RPMI-1640 medium and incubated further for 3 days. Vascular endothelial growth factor (VEGF), thalidomide, purified PKAc, and concentrated serum-free conditioned media containing total secreted proteins including ECPKA from HCT-116 and LnCap cells were embedded in circular 0.45% methylcellulose (Sigma, St. Louis, MO, USA) discs formed on glass cover slips, and air-dried. The discs were lifted from the cover slips and placed directly onto CAM surface of the chicken embryos and incubated at 37°C for approximately 72 h. New blood vessels growth or vascular zones on CAM tissue were imaged and the area of treatment under the methylcellulose disc was scored for new vessels and capillaries growth using the ImageJ software suite (National Institute of Health, Bethesda, MD, USA). Studies were conducted in triplicate (3 chick embryos per treatment condition) and standard deviations were evaluated.

Immunoblotting

Serum-free conditioned media of HCT-116 and LnCap cells were collected and concentrated using Amicon column (Millipore, Billerica, MA, USA). Approximately 20 µg of total proteins were electrophoresed on 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. Immunoblot detection was performed with the corresponding rabbit antiserum or mouse monoclonal antibody specific against the various PKAc isozymes (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using an enhanced chemiluminescence detection kit and exposure to photographic film.

PKA and Activity Assay

Purified C subunit of PKA was purchased from Promega (Madison, WI, USA). PKA activity was measured using the Omnia Lysate PKA Assay Kit (Invitrogen/BioSource, Carlsbad, CA, USA) according to manufacturer's specifications.

RESULTS

The identification of ECPKA in the serum of cancer patients and in conditioned media of cultured cancer cells raises questions about its function in the tumorigenic development of cancer. As cancer growth and metastasis are angiogenesis-dependent (Duffy, McGowan, & Gallagher, 2008), we asked here whether ECPKA might be involved in tumor angiogenesis. We applied the chicken embryo chorioallantoic membrane (CAM) assay to determine the role of PKAc, and ECPKA from the conditioned media of cultured colon (HCT-116) and prostate (LnCAP) cancer cells, on new blood vessels formation.

We first examined the effects of the angiogenic vascular endothelial growth factor (VEGF) (Roy, Bhardwaj, & Yla-Herttuala, 2006) and found distinct enhancement of new vessels and capillaries growth by VEGF compared to either untreated or serum-free RPMI-1640 media-treated control (Figure 1). The anti-angiogenic drug thalidomide (Richardson, Hideshima, & Anderson, 2002), in contrast, inhibited the formation of new blood vessels (Figure 1), characterized by fewer major vessels and capillaries under the area of treatment. The amount of new blood vessels growth was also quantified using the ImageJ software suite, focusing on the area of treatment under the methylcellulose disc, which showed approximately 50% inhibition of angiogenesis by thalidomide (Figure 2A). Exposure of the chicken embryo to various concentrations (10, 50, and 100 ng) of PKAc resulted in the inhibition of new blood vessels growth as evident by the lack of capillaries, compared to untreated, media alone, or VEGF-treated controls (Figure 3). Inhibition of new

blood vessels growth by PKAc was not dose-dependent. Furthermore, PKAc mediated inhibition of angiogenesis seemed to be comparable to that of thalidomide (Figures 2A and 3).

Since activated intracellular PKA activity is normally terminated by either the hydrolysis of cAMP or the inhibition of the kinase by PKI (Dalton & Dewey, 2006), we asked whether co-treatment of chicken embryo with PKI might reverse the inhibition of new blood vessels growth by PKAc. Results in Figure 2A showed that PKI alone did not have an effect on angiogenesis, but the inhibition by PKAc was partially reversed by PKI.

We next examined the effects of ECPKA obtained from the serum-free conditioned media of the cultured colon cancer HCT-116 and the prostate cancer LnCaP cell lines. The media were concentrated and constitutively active PKA activities were detected by a fluorescent peptide substrate, and the presence of ECPKA in the conditioned media of HCT-116 and LnCap cells was determined by Western blotting (Figure 2B and C). Isozyme-specific antibodies against the C subunit of PKA showed that Cα and Cβ (Figure 2C), but not Cγ (data not shown), were found in the conditioned media of HCT-116 and LnCAP cells. Addition of various concentrations (10, 50 or 100 μg) of total secreted proteins from the conditioned media of either HCT-116 or LnCap cells, which contained ECPKA, to the chicken embryo caused reduction of new blood vessels growth in a dose dependent-manner (Figure 3). The extent of inhibition was comparable to the effects of thalidomide and PKAc. However, unlike PKAc, inhibition of new blood vessels growth by ECPKA from either HCT-116 or LnCap cells was not reversed by PKI (data not shown). These results showed that PKAc, and ECPKA of conditioned media of cultured cancer cells, inhibited new blood vessels growth in the CAM assay.

It is noteworthy that application of ECPKA-positive serum from cancer patients onto CAM induced rapid death to the chick embryo within 10-15 h (data not shown), possibly resulting from the cross-interaction of patients' antibodies with chicken embryonic antigens. These results hampered further direct examination of clinical serum sample using this assay.

DISCUSSION

Release of the free C subunit of PKA into the extracellular milieu including the serum of cancer patients is an intriguing phenomenon (Cho, Park, et al., 2000; Cvijic, et al., 2000; Kita, et al., 2004; H. Wang, et al., 2007). We speculate that the aberrant and ubiquitous secretion of ECPKA by cancer cells into the serum of cancer patients serves to fuel the growth of the cancer either by promoting angiogenesis or tumor metastasis. However, our results in this study showed paradoxically that purified PKAc and ECPKA in the conditioned media of cultured cancer cell inhibited angiogenesis by the CAM assay (Figures 1 and 3). Therefore, it is counterintuitive that cancer cells release a constitutively active protein kinase that inhibits angiogenesis, which might actually impede their growth and metastatic potential, thus compromising tumorigenesis.

While the role of the intracellular PKA holoenzyme in cell growth has been widely investigated (Chin, et al., 2002), little is known, however, regarding the function of this recently identified secreted ECPKA. It has been shown that enhanced activity of the intracellular PKA promotes endothelial tube formation, thus facilitating angiogenesis (Hashimoto, Miyakoda, Hirose, & Mori, 2006). In contrast, it has also been shown that activation of intracellular PKA causes the phosphorylation of the transcriptional repressor Id1 (a major contributor to angiogenesis) (Volpert, et al., 2002), and disrupts its nucleocytoplasmic shuttling, thus inhibiting angiogenesis (Nishiyama, et al., 2007). In addition, either overexpression of the C subunit gene of PKA or pharmacological activation of PKA induces the death of endothelial cell by

apoptosis (Kim, Bakre, Yin, & Varner, 2002), hence inhibiting angiogenesis. Though others and we have shown that in normal individuals, levels of ECPKA activity in the peripheral blood are negligible (Cho, Park, et al., 2000; Cvijic, et al., 2000; Kita, et al., 2004; H. Wang, et al., 2007), but we have not explored whether ECPKA might be secreted when a system is perturbed in normal subject. Therefore, it is conceivable that intracellular PKA has angiogenic function during development that is distinct and independent of the action of ECPKA. While we did not measure endothelial cell death in this study, it is possible that ECPKA inhibits angiogenesis by inducing endothelial apoptosis. Moreover, overexpression of the Cα subunit gene of PKA has been shown to increase the production of ECPKA (Cho, Park, et al., 2000), which might account for the inhibition of angiogenesis noted in our study, and is consistent in part with the observed endothelial cell death.

We currently do not understand the underlying reasons for the release of the free C subunit of PKA in cancer. Once secreted, does ECPKA play the role of a driver gene in cancer development, or is it merely a passenger gene (Greenman, et al., 2007), which confers no growth advantage, amidst the myriad of genes targeted for alterations? For one, we speculate that the aberrant secretion of ECPKA might be an unintended consequence of tumor progression. Resultantly, the release of ECPKA into the serum of cancer patients elicits an autoimmune response, because PKA is normally localized inside the cell and the extracellular C subunit might be antigenic. This hypothesis is supported by the discovery of autoantibody against ECPKA in the serum of cancer patients (Nesterova, et al., 2006). We surmise that the production of autoantibody against ECPKA rapidly neutralizes its activity, hence preventing it from inhibiting angiogenesis and acting on its extracellular substrates or targets.

What is the pathogenic mechanism underlying ECPKA secretion in cancer? In human, there are three C subunit isoforms, Cα, Cβ, and Cγ, which have different biochemical and functional properties (Skalhegg & Tasken, 2000). We showed here that Cα and Cβ are the predominant isozymes that constitute the ECPKA secreted by HCT-116 and LnCAP cells (Figure 2C). The level of Cγ was not detectable in these conditioned media (data not shown). Therefore, Cα and Cβ are presumably the major isozymes released in cancer. Furthermore, the R and C subunits of PKA are normally present in a 1:1 stoichiometric ratio (Skalhegg & Tasken, 2000), hence there is little free PKAc intracellularly. It is conceivable that either the Cα or the Cβ subunit isozyme is overexpressed, or that the R subunits are downregulated in cancer, thereby resulting in excess free PKAc that are not associated with the R subunits, thus leading to their release out of the cells. Alternatively, mutations in the C subunit genes may result in the failure to retain these proteins in the cells, thus leading to their secretion into the extracellular milieu. Therefore, sequencing the C subunit genes in cancer might yield insights into their susceptibility to genetic alterations that predisposed them to secretion.

The ubiquitous nature of the release of ECPKA and its potential application as biomarker in cancer, and whether the aberrant release of ECPKA is just a bystander effect or that it might have important function in cancer development warrant further investigation.

Conflict of interest

There is no conflict of interest for any of the authors.

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FIGURE LEGENDS

Figure 1. Angiogenic activity assessed by the chick embryo CAM assay. Methylcellulose discs loaded with RPMI-1640 media (Control), VEGF (200 ng), or thalidomide (400 ng), were placed on CAM surface of chick embryo. Photomicrograph view of the CAM at the site of the disc approximately 72 hr following drug treatment showing either induction or inhibition of allantoic blood vessels growth. Photomicrographs were obtained from representatives of triplicate experiments.

Figure 2. **A**. Effects of PKAc and PKI on allantoic vessels growth. Methylcellulose discs were embedded with either PKAc (10 ng), PKI (1 μM), or PKAc + PKI. Effects of PKAc and PKI on blood vessels growth were compared to serum-free RPMI-1640 media-treated control, VEGF (200 ng) and thalidomide (400 ng). **B**. Kinase activity of ECPKA of HCT-116 and LnCAP. Conditioned media of HCT-116 and LnCAP cells were collected, concentrated, and 10 μg of total protein from the concentrates were used for the PKA activity assay according to manufacturer's specification, and compared to PKAc control (10 ng). (**B** and **C**, results are means ± S.E. of triplicate experiments and statistical significance was assessed using Student's T-test; asterisk, $P<0.05$). **C**. Western blot analysis of ECPKA. Conditioned media containing ECPKA from HCT-116 and LnCAP cells were probed with isozyme specific antibodies against either the Cα or the Cβ subunit of PKA, or with a pan-PKAc antibody.

Figure 3. Angiogenic response to PKAc and ECPKA by the CAM assay. Effects of various concentrations of either PKAc (10, 50, or 100 ng) or ECPKA obtained from conditioned media of HCT-116 (50 or 100 μg of total secreted protein) and LnCAP (10, 50, or 100 μg of total secreted protein) cultured cancer cells, on blood vessels growth in chick embryo CAM. Number of blood vessels at the methylcellulose disc and CAM boundary were compared with untreated, serum-free media, VEGF and thalidomide controls. Photomicrographs were obtained from representatives of triplicate experiments. Histogram showed quantitative data from the experiments in triplicate and results are means ± S.E. of triplicate experiments and statistical significance was assessed using Student's T-test; asterisk, $P<0.05$.

Figure 1

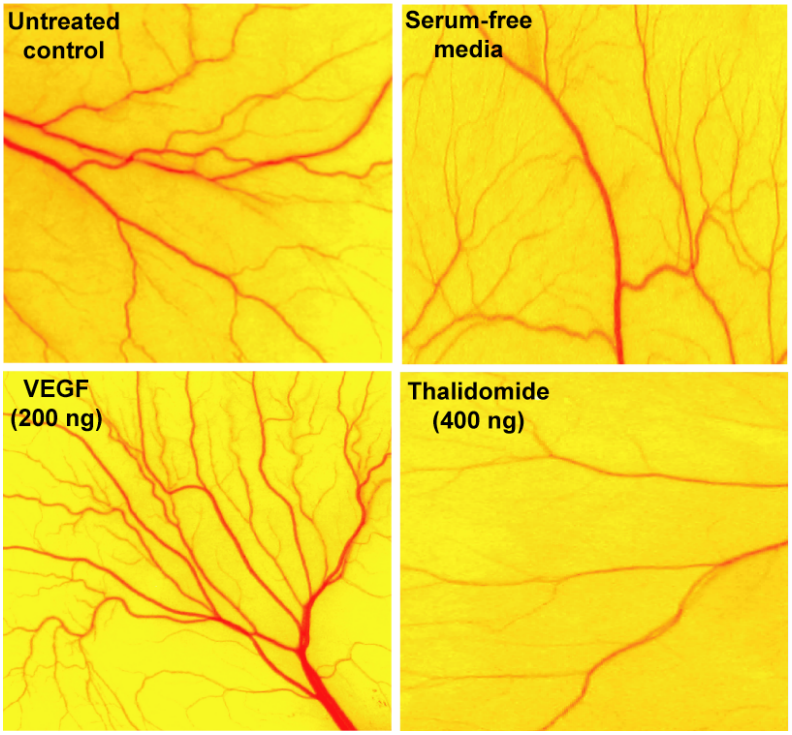

Untreated control
Serum-free media
VEGF (200 ng)
Thalidomide (400 ng)

Figure 2

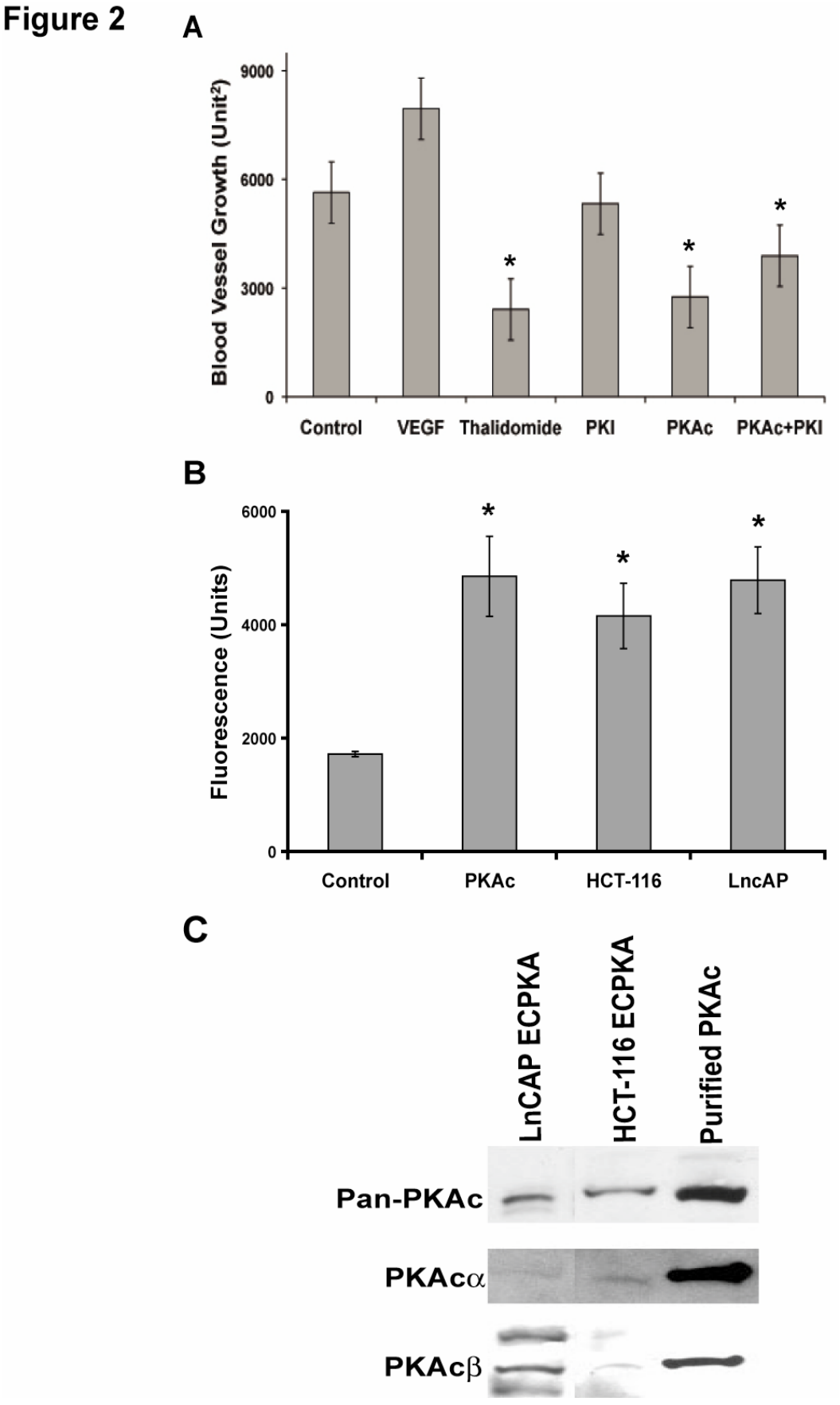
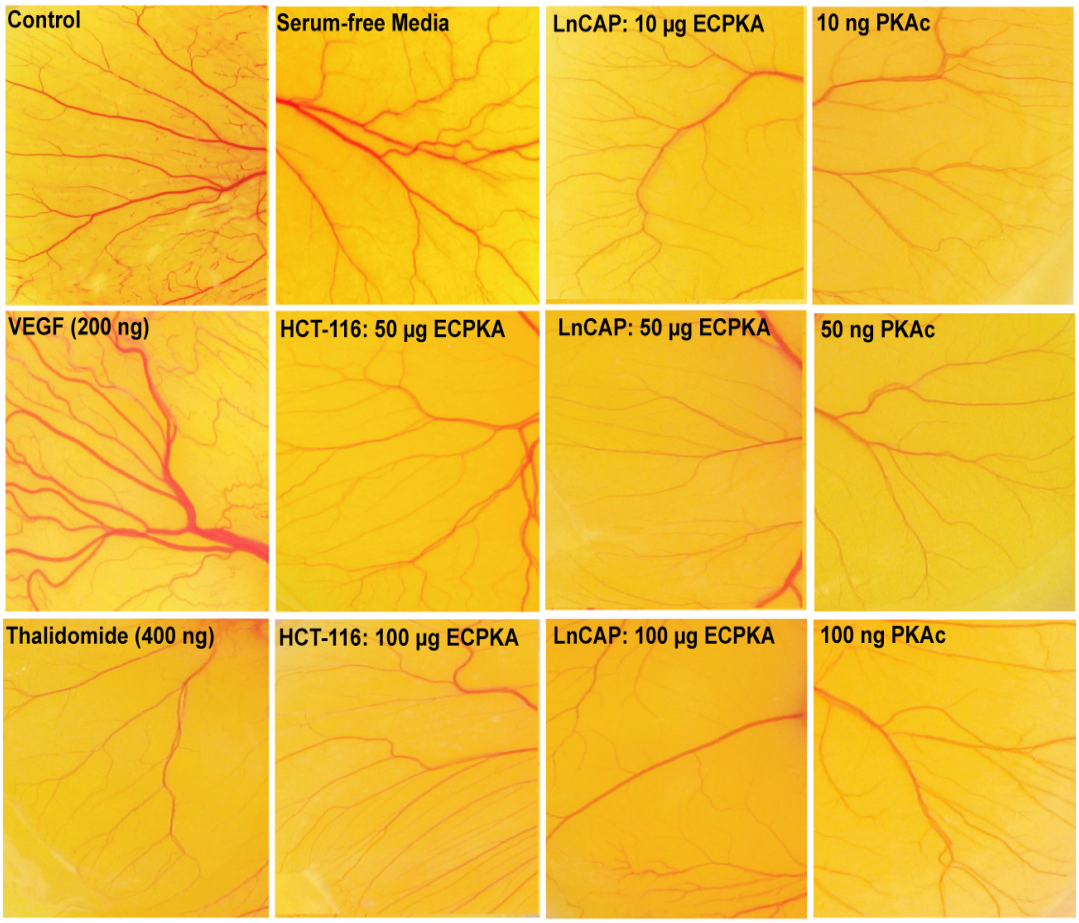
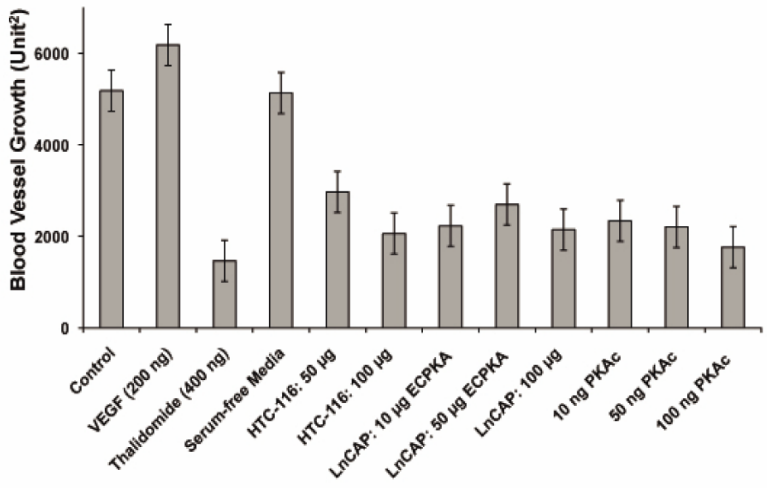

A
Blood Vessel Growth (Unit²)
9000
6000
3000
0
Control
VEGF
Thalidomide
PKI
PKAc
PKAc+PKI
B
Fluorescence (Units)
6000
4000
2000
0
Control
PKAc
HCT-116
LncAP
C
LnCAP ECPKA
HCT-116 ECPKA
Purified PKAc
Pan-PKAc
PKAcα
PKAcβ

Figure 3


Control
Serum-free Media
LnCAP: 10 µg ECPKA
10 ng PKAc
VEGF (200 ng)
HCT-116: 50 µg ECPKA
LnCAP: 50 µg ECPKA
50 ng PKAc
Thalidomide (400 ng)
HCT-116: 100 µg ECPKA
LnCAP: 100 µg ECPKA
100 ng PKAc


Blood Vessel Growth (Unit²)
6000
4000
2000
0
Control
VEGF (200 ng)
Thalidomide (400 ng)
Serum-free Media
HTC-116: 50 µg
HTC-116: 100 µg
LnCAP: 10 µg ECPKA
LnCAP: 50 µg ECPKA
LnCAP: 100 µg
10 ng PKAc
50 ng PKAc
100 ng PKAc

Chapter III: Manuscript 2

MOLECULAR REGULATION OF ADIPOGENESIS BY PRIEURIANIN

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ABSTRACT

Adipogenesis or the development of fat cells from preadipocytes, is a key differentiation process that has been extensively studied in the past decade. Adipocyte differentiation involves temporally regulated genes, controlled by a well-defined set of transcription factors, CCAAT/enhancer-biding proteins (C/EBP) β, C/EBPδ and C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ). Zinc finger-containing transcription factors have been implicated to play an important role during cell differentiation and development. Previously, we have identified a novel anti-obesity natural product, prieurianin by high-throughput screen. Gene expression profiling showed that prieurianin suppresses the expression of a number of genes involved in fat metabolism. Here we show that Zfp68 (zinc finger protein 68), a previously identified transcriptional repressor, is prominently induced by prieurianin as an immediate-early gene. First, Zfp68 inhibited the transcriptional activity of the adipogenesis master regulators including the CCAAT/enhancer binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor γ (PPARγ). Second, overexpression of the zfp68, nuclear localized protein, attenuated the OP9 cell potential to differentiate into mature adipocytes. These results suggest for the first time that Zfp68, a member of KRAB zinc finger family may act as a novel transcriptional repressor of adipogenesis.

INTRODUCTION

Metabolic syndrome is becoming a global epidemic due to the rapid rise in the incidence of overweight and obese individuals worldwide. Obesity is an energy balance disorder, in which nutrient intake chronically exceeds energy expenditure often characterized by an overgrowth of adipose tissue and excess storage of triglyceride (Dvorak, Starling, Calles-Escandon, Sims, & Poehlman, 1997). Excess adipose tissue can be a result of both hypertrophy (increased lipid accumulation) and hyperplasia (increased proliferation/differentiation) of adipocytes (Kahn, 1992). The primary function of adipose tissue is to store energy in the form of triglycerides during periods of energy excess and to release the energy during fasting or starvation as free fatty acids and glycerol. Because adipose tissue serves as a critical integrator of glucose homeostasis, understanding of adipocyte biology is crucial for understanding the pathophysiological basis of obesity and its associated diseases (Rosen & Spiegelman, 2006).

The alarming rate of increase in obesity, largely due to sedentary lifestyle coupled with overconsumption of energy-rich foods, has significantly raised the risk and incidence of associated comorbidities such as diabetes, hypertension, cardiovascular and metabolic diseases (Bray & Bellanger, 2006). Despite the wealth of information about the hormonal and transcriptional mechanisms that regulate energy metabolism, there are currently only two drugs approved by the FDA for the treatment of obesity, orlistat and sibutramine. These drugs have limited efficacies and side effects are commonly reported, which are further confounded by diminishing response in long-

term treatment (Fernstrom & Choi, 2007; Li, Maglione, et al., 2005). Hence, pharmacotherapy of obesity is an unmet need, and the development of new and effective anti-obesity drugs is essential. Therefore, increasing our understanding of the mechanisms that regulate energy homeostasis is crucial and may lead to novel obesity therapies.

Previously, we identified prieurianin in a high-throughput screen for novel topoisomerase I inhibitors. Prieurianin has been shown to be an insect feeding deterrent by acting on the central nervous system (Sarker, Savchenko, Whiting, Sik, & Dinan, 1997). Taking advantage of its anti-feedant property and potential application in human for the treatment of obesity, we examined the anti-obesity effects of prieurianin *in vivo* in genetically obese leptin-deficient *ob/ob* and leptin-receptor deficient *db/db* mice, diet-induced obese (DIO) and *Cc1-/-* mice. We observed that prieurianin significantly inhibited the accumulation of fat droplets in adipocytes following differentiation in a dose-dependent manner, when compared to untreated - control or vehicle - treated cells (unpublished data). Taken together these results showed that prieurianin is an effective anti-adipogenic as well as anti-obesity compound causing weight loss in mice by various mechanisms.

Using DNA microarray, we examined the effects of prieurianin on the expression profiles of preadipocytes undergoing differentiation. Our results showed, as expected, that the block of differentiation by prieurianin was accompanied by the inhibition of expression of genes that are involved either directly or indirectly in fatty acid metabolism, which include transcription factors and hormone receptors such as C/EBPα, C/EBPβ, and PPARγ (unpublished data). The changes in the expression of the known regulators of adipogenesis found in our microarray study largely overlapped with those identified previously (Burton, Nagarajan, Peterson, &

McGehee, 2004). These results showed that prieurianin is a bona fide anti-adipogenic compound, which inhibits preadipocyte differentiation into adipocytes by downregulating or inhibiting the expression of critical adipogenic factors that regulate differentiation (Figure 1B). Interestingly, among the small cluster of immediate early genes induced by prieurianin include the zinc finger protein 68 (Zfp68).

Zfp68 also called KRAZ2 (KRAB-containing zinc finger proteins) belongs to the C_2H_2 superfamily of zinc finger proteins. The Cys_2His_2 (C_2H_2 or Krüppel) type zinc finger proteins (ZFPs) are a class of well-defined eukaryotic DNA-biding proteins and represent the single largest class of transcription factors (Bellefroid, et al., 1989). About one third of the large subclass of C_2H_2 Krüppel-like zinc finger proteins contains the evolutionary conserved Krüppel-associated box (KRAB) domain, which is characterized by a 75 amino acid conserved sequence present at the N-terminus of the zinc fingers (Mark, Abrink, & Hellman, 1999). When tethered to a heterologus DNA binding domain (DBD), KRAB acts as a transcriptional repressor (Witzgall, O'Leary, Leaf, Onaldi, & Bonventre, 1994). KRAB domain consists of KRAB A and KRAB B boxes. KRAB A, encodes a 45 amino acid core present within the 75 amino acid conserved sequence and it is typically necessary for repression (Vissing, Meyer, Aagaard, Tommerup, & Thiesen, 1995). Roles for Krüppel-type zinc finger genes have been suggested in the regulation of normal cell growth, differentiation, embryogenesis and tumorigenesis (Rue, Kim, Jun, & Kim, 2001).

Zfp68 is found to be ubiquitously expressed in all mouse tissue tested (Matsuda, et al., 2001). Zfp68 cDNA (2261 nt) contains an open reading frame (nt 308-2128) that encodes a polypeptide of 606 amino acids with a molecular mass of 71.2 kDa (Agata, et al., 1999). It contains KRAB-A domain followed by the KRAB-B domain at the N-

terminus and nine repeated zinc finger domains located in the C-terminus. Mechanistically little is know about how Zfp68 regulates gene expression, but it was previously shown that Zfp68 may be a transcriptional repressor and the KRAB-A domain is responsible for transcriptional repression (Agata, et al., 1999). The regulation of zfp68 is also understudied. We are the first to report in a DNA microarray analysis that Zfp68 is prominently induced by prieurianin, a novel anti-obesity drug, suggesting that zfp68 plays an important role in adipocyte physiology. Zfp68 is predominantly known for its association with the corepressor KAP-1 (KRAB-associated protein-1) (Agata, et al., 1999), which interacts with heterochromatin protein 1 (HP1) (Matsuda, et al., 2001), thus raising the possibility that Zfp68 could silence the transcriptional activity of C/EBPs and PPARγ, thereby inhibiting preadipocytes differentiation into adipocytes. Since expression of Zfp68 correlates with repression of C/EBPs, known precursors of adipocyte differentiation, it is reasonable to propose that Zfp68 plays an important role as a negative regulator of adipogenesis. The goal of our studies was to characterize the transcriptional regulation of adipogenesis by prieurianin and determine the anti-adipogenic effect of Zfp68.

MATERIAL AND METHODS

Cell culture, chemicals

Bone marrow-derived mouse stromal OP9 cells (Wolins, et al., 2006) (a gift of Dr. Perry Bickel, University of Texas Health Science Centre, Houston) were cultured at 37°C in OP9 propagation media: MEM-α with 20% FBS (Premium Fetal Bovine Serum, catalog no. 14-051Q, Atlanta Biologics), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. 3T3-L1 (L1) cells, obtained from the American Type Culture Collection were cultured in 3T3-L1 propagation media: DMEM with 10% bovine serum (Invitrogen, catalog no. 16170-078), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Differentiation

OP9 were grown to confluence and then cultured for 2 additional days in OP9 propagation media. The cells were then cultured up to 3 more days in serum replacement (SR) media: MEM-α (HyClone, SH 3026501) with 15% KnockOut Serum Replacement (Invitrogen, catalog no. 10828-028), 100 U/ml penicillin, and 100 µg/ml streptomycin, and then replenished in the propagation medium.

3T3-L1 cells were differentiated as previously described (Student, Hsu, & Lane, 1980). In brief, the L1 cells were grown to confluence and then cultured for 2 additional days in 3T3-L1 propagation media. Cells were then cultured 2 days in DM1: DMEM with 10% FBS, 175 nM insulin, 0.25 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml

streptomycin. The cells were then replenished with DM2: DMEM with 10% FBS, 175 nM insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. At that point, 3T3-L1 cells were maintained in 3T3-L1 adipocyte media.

Lipid accumulation in adipocytes was visualized by staining with Oil Red-O or Nile Red (Sigma).

Plasmid, Transfection and Reporter Assays

C/EBP and PPARγ response-elements reporter plasmids were constructed by ligating oligonucleotides containing either three C/EBP- or PPARγ-response elements in tandem into the pGL3-Luc reporter expression vector (Fig. 5A) and sequence-verified. The mouse PPARγ_2 gene promoter was amplified as previously described (Zhu, et al., 1995) with primers: forward (5'-CTAGCTAGCGCTCCCAC-GTTAGCAGTTTGGCAC-3'and reverse 5'-CCCAAGCTTCTTGCAGCAACATCAGGAATGGAC-3') including *Nhe* I and *Hind* III sites, subcloned into pGL3-basic vector (Promega) and sequence verified.

The melanoma Mel501 cells (American Type Culture Collection) or OP9 cells were transfected with expression constructs for C/EBPs, and PPARγ and contransfected with zfp68 expression construct using the Lipofectamine 2000 transfection reagent (Invitrogen). Equal amounts of DNA were used for all transfection by adding the appropriate amount of salmon sperm DNA and the *Renilla* luciferase reporter as internal control. Relative luciferase activities were determined 24 h following transfection and normalized to the *Renilla* luciferase activity. All transfection experiments were performed in triplicates.

RT-PCR

Total RNA was isolated from 3T3-L1 cells using RNA Isolation Kit (Qiagen). First strand cDNA was synthesized using 1 μg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen), followed by RT-PCR using β-actin as internal control. The primer set used: forward 5'-GGGTCATGGATTGTAGAAGC-3', reverse 5' GGATTTCCCTGGTATCAGAC-3'.

Western Blots

Cell lysates prepared using 1% NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl , 1% NP-40, Protease Inhibitor Cocktail (Roche)) were separated by SDS-PAGE and transferred to PVDF membranes (162-0177; Bio-Rad). Proteins were detected using antibodies specific for C/EBPβ (sc-7962; Santa Cruz), C/EBPα (sc-61; Santa Cruz), PPARγ_2 (P0744; Sigma), and β-actin (A3854; Sigma). Mouse or rabbit bound horseradish peroxidase-coupled secondary antibody (Amersham Bioscience) was visualized with enhanced chemiluminescence HyGLO detection reagent (Denville, E-2500).

Microarray analysis

3T3-L1 cells were differentiated in the presence of prieurianin (2 μM) in a time course analysis from 0, 0.25, 0.5, 1, 2, 3, 5, 9, 12, 15, 24, 36, 48, 96, 144, 192, 240, to 288 hrs, and compared to untreated and vehicle-treated controls. Total RNA was purified at the indicated time, then labeled and hybridized to the Mouse OneArray (Phalanx Biotech Group, Palo Alto, CA), and analyzed as previously described

(Zheng, et al., 2002). Raw microarray data is available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih. gov/geo/) under series accession no. GSE15018).

Statistical analysis

Commercially available software package (PRISM, GraphPad Software, Inc., La Jolla, CA) or the data analysis add-in for Microsoft Excel were used for performing statistical analysis. Either single factor ANOVA or Student's t-tests were used for all analysis. P values of less than or equal to 0.05 were considered statistically significant.

RESULTS & DISCUSSION

Advances in genomics, in particular microarray-based expression profiling, have provided scientists with a number of new candidate genes whose expression in adipose tissue is regulated in obesity (Dahlman & Arner, 2007). Global gene expression changes following the onset of differentiation exhibited a distinct induction and suppression of gene expression by prieurianin (Figure 1A). It has been speculated that gene expression within the first 24 hrs following induction of differentiation and mitotic clonal expansion are considered a prerequisite to adipogenesis (MacDougald & Lane, 1995). Our results showed, as expected, that the blocking of differentiation by prieurianin is accompanied by the inhibition of the expression of genes that are involved either directly or indirectly in fatty acid metabolism, which include transcription factors and hormone receptors such as C/EBPα, C/EBPβ, and PPARγ. The changes in the expression of the known regulators of adipogenesis found in our microarray study largely overlapped with those identified previously (Burton, et al., 2004) (Figure 1B). It is important that Krox20 (EGR1), previously identified to be necessary for adipogenesis (Chen, Torrens, Anand, Spiegelman, & Friedman, 2005), though induced early transcriptionally during differentiation (Figure 1B), its expression persisted, despite the inhibition of differentiation by prieurianin, thus raising the question about its role in adipogenesis under these conditions.

DNA microarray also demonstrated the temporal pattern of early, intermediate, and late response gene clusters, according to their relative time of induction (Figure

1C). Early response genes were activated transiently and rapidly (within minutes) in response to a prieurianin treatment. The onset of induction of the intermediate response genes occurred hours after drug treatment, while the late response genes were activated last, induced, or repressed within days. Our microarray analysis revealed many gene expression changes following treatment with prieurianin, including the master regulators of adipogenesis. However, we focus our attention in subset of early response genes, since early response genes often function to multiply and propagate the initial gene activation process, and they are responsible for setting the stage for long-term changes in adipogenesis. Interestingly, among the small cluster of immediate early genes induced by prieurianin include the zinc finger protein 68 (Zfp68) (Figure 1D). Zfp68 expression was induced up to 15 fold within 15 min following prieurianin treatment, thus suggesting that it may play a critical role in the inhibition of adipogenesis. A subset of expression changes was verified by polymerase chain reaction with reverse transcription (RT-PCR) from OP9 stromal cells treated with prieurianin in a time course (Figure 2). We observed modest induction of Zfp68 by prieurianin in a dose and time-dependent manner, which is consistent with the microarray data. The increase in Zfp68 expression at “0” time point however, perhaps may be explained as the time interval between the treatment and the sample collection.

Zfp68 or KRAZ2 (KRAB-containing zinc finger proteins) is a member of the superfamily of zinc finger protein. Zfp68 cDNA (2261 nt) contains an open reading frame (nt 308-2128) that encodes a polypeptide of 606 amino acids with a molecular mass of 71.2 kDa (Agata, et al., 1999). It contains KRAB-A domain followed by the KRAB-B domain at the N-terminus and nine repeated zinc finger domains located in the C-terminus (Figure 3A). The KRAB domain is encoded by many transcription

factors (Figure 3B). It has been shown that Zfp68 may be a transcriptional repressor and the KRAB-A domain may be responsible for transcriptional repression (Agata, et al., 1999). Zfp68 was found to be ubiquitously expressed in all mouse tissue tested (Matsuda, et al., 2001)

In an attempt to investigate the anti-adipogenic effect of zfp68, we first wanted to verify its distribution. Many C_2H_2 type of zinc finger proteins have been demonstrated to localize in the nucleus and to act as transcription repressors (Skapek, et al., 2000). To determine whether Zfp68 is localized in the nucleus, we transiently expressed a fusion protein between Zfp68 and green fluorescent protein in HEK293A cells. Twenty-four hours after the transfection, the cells were visualized with a fluorescence microscope after labeling with DAPI. Zfp68-GFP protein is found in the nucleus (white arrows) when overexpressed in these cells (Figure 4), which is in agreement with previous reports (Matsuda, et al., 2001).

Zinc finger DNA-binding proteins containing KRAB domains usually show a powerful ability to repress gene expression. Since Zfp68 was induced by prieurianin, a potential anti-obesity drug, we reasoned that Zfp68 might be a master repressor of the transcriptional regulators of adipogenesis. Moreover, the expression of C/EBPα, C/EBPβ, and PPARγ are thought to be crucial for the regulation of preadipocyte differentiation. To determine whether Zfp68 transcriptionally regulates the expression of key regulatory genes of adipogenesis, we cotransfected F501 melanoma cells with either C/EBPα, C/EBPβ, or PPARγ cDNA expression vectors and their corresponding response element-driven luciferase reporter gene (Aperlo, Pognonec, Saladin, Auwerx, & Boulukos, 1995) (Figure 5A) in the presence or absence of Zfp68. Zfp68 alone showed no significant effect on the basal activity of the response element reporters, however, cotransfecting Zfp68 with C/EBPα, C/EBPβ (Figure 5B), or

PPARγ (Figure 5C), inhibited the transcriptional activity of C/EBPα and C/EBPβ, and PPARγ from their respective response-element driven promoters, suggesting a direct role of Zfp68 in the repression of the transactivation potential of these master regulators of adipogenesis. Zfp68 inhibits transcription through its association with the corepressor KAP-1 (KRAB-associated protein-1) (Agata, et al., 1999), which interacts with heterochromatin protein 1 (HP1) (Matsuda, et al., 2001)

Since Zfp68 plays a critical role in the transcriptional repression of adipogenesis induced by prieurianin by inhibiting C/EBPα-, C/EBPβ-, and PPARγ-regulated gene expression during preadipocytes differentiation it is noteworthy to further elucidate the anti-adipogenic effect of Zfp68 on adipocyte differentiation. To test this possibility directly, we transfected OP9 stromal cells with either empty vector or Zfp68 and subsequently induced them to differentiate into adipocytes. After three days, cells were stained with Nile Red (9-diethylamino-5H-benzo [alpha] phenoxazine-5-one), a vital stain for the detection of intracellular lipid droplets by fluorescence microscopy. As shown in (Figure 6A), compared to either vector or untransfected control, Zfp68-overexpressing cells showed a decrease in intracellular lipid accumulations as evidenced by reduced Nile Red or Oil Red O staining. That lead us to the conclusion, that overexpression of Zfp68 attenuated the OP9 cell potential to differentiate into mature adipocytes.

Transcription factors are necessary for adipogenesis. If Zfp68 is an early transcriptional repressor of adipogenesis, it should inhibit the expression of the key adipogenic proteins. Hence, next we examined expression of molecular markers of terminal differentiation at the protein level by Western blotting and observed and inhibition of endogenous C/EBPα and C/EBPβ, and PPARγ genes (Figure 6B).

Adipogenesis involves a temporally regulated set of genes (Figure 7), therefore to investigate and characterize the regulation of Zfp68 expression by prieurianin it is crucial to understand the underlying transcriptional network. Over the past two decades, attention has centered on the role of the nuclear receptor PPARγ and the members of C/EBP family of transcription factors in adipogenesis (Rosen & MacDougald, 2006). In light of our data, here we conclude that Zfp68 is one of the immediate early genes that mediate the transcription inhibition of adipogenesis by prieurianin. Although more studies are required to examine the effects and mechanisms of Zfp68 *in vitro* and *in vivo*, the elucidation of Zfp68 signaling events during adipogenesis might be an important area for future investigations.

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FIGURE LEGENDS:

Figure 1. Genome-wide gene expression profiling shows that prieurianin disrupts the transcriptome of adipogenesis. Dendrograms of gene expression changes during differentiation of L1 preadipocytes showing specific alterations induced by prieurianin compared to DMSO or untreated control. **A**, Specific clusters of genes whose expression were either induced or inhibited by prieurianin. **B**, Attenuated expression of C/EBPα, C/EBPβ, and C/EBPδ, and PPARγ, transcriptional regulators of adipogenesis . Neither EGR1 nor EGR2 expressions were affected by prieurianin. **C**, Distinct temporal changes in gene expression were clustered into early, intermediate and late responses during preadipocyte differentiation into adipocytes. **D**, Dendogram of the expression profile of zfp68 in response to treatment with prieurianin. The complete data set is available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under series accession no. GSE15018).

Figure 2. Regulated expression of zfp68 by prieurianin during adipogenesis. OP9 cells were treated with either DMSO (vehicle) or prieurianin (2, 4 or 6 μM) during differentiation. Total RNA was isolated at various times, and mRNA levels of zfp68 were estimated by reverse transcription PCR RT-PCR, with β-actin as the reference gene. Values are means ± S.E.M. from three independent experiments: *$p<0.05$, # $p<0.001$ versus vehicle treated cells at each time point.

Figure 3. Structure and amino acid sequence of zfp68 (KRAZ2). **A,** Schematic representation of the structure of the zfp68 protein *(upper panel)*. **B,** Comparison of the KRAB domains from various zinc-finger proteins. The KRAB-A and KRAB-B domains and the multiple zinc finger domains are indicated by *black*, *gray*, and *shaded boxes,* respectively. The predicted amino acid sequences of the zfp68 protein are indicated in each *lower portion*. The KRAB-A and KRAB-B domains are *double* and *single underlined*, respectively. The multiple zinc finger domains are *boxed*, and cysteine and histidine residues are highlighted with *black. B,* alignment of the amino acid sequences of the multiple KRAB domains. The KRAB domains of 15 zinc finger proteins including KOX1 are aligned with the zfp68 sequence. The consensus sequence is shown at the *bottom*, and conserved residues corresponding to the consensus are shown on a *black background*. Data base accession numbers are as follows: KOX-1, X52332; ZFP90/NK10, X79828; ZNF91/HTF10, L11672; ZNF140, U09368; ZNF133, U09366; ZNF85,U35376; mKID-1, L77247; rKID-1, M96548; ZNF141, L15309; ZNF90, M61870; ZNF157, U28687; ZNF43, X59244; ZNF7, M29580; ZNF177, U37263; and ZNF45,L75847. Figure adapted from (Agata, et al., 1999).

Figure 4. Nuclear localization of zfp68. HEK293A cells were transiently transfected with either an empty vector (upper panel) or GFP-zfp68 fusion protein (lower panel). The green fluorescence signal from the GFP fusion construct was monitored using a fluorescence microscopy. Nuclei were counterstained with DAPI (blue signal).

Figure 5. Mechanism of CEBPRE and PPRE transactivation. The F501 melanoma cells at 70% confluency were contransfected with CEPBRE (**A**) and PPRE reporter plasmids (**B**) and other expression plasmids, using Turbofectin (Origene). Cell luciferase activity was assayed after 24h using Luciferase Assay System (Promega). Results are the means ± SE of triplicate experiments. Statistics were conducted as student t-test. Asterisk, p,<0.05 vs. vector; hash p<0.05 vs, the respective C/EBPα-, C/EBP β-, PPARγ -transfected controls. Results are means ± S.E.M.., normalized to *Renilla* luciferase activity.

Figure 6. Overexpression of zfp68 attenuated adipocyte differentiation. **A** and **B**, OP9 cells were untransfected (left panel) or transfected with either empty vector (middle panel) or FLAG-zfp68 fusion protein (right panel) followed by induction to differentiate into adipocytes. Following differentiation Nile Red and Oil Red O staining was performed to access for lipid accumulation (**A**) or the cells were harvested at the indicated time points and immunoblot analysis was performed using C/EBPα, C/EBPβ and PPARγ antibodies with β-actin serving as internal control as described under "*Material and Methods*" (**B**).

Figure 7. Mechanism of transcriptional regulation of adipogenesis by prieurianin. The expression of peroxisome proliferator-activated receptor γ (PPAR γ), the core of transcriptional cascade that regulates adipogenesis, is regulated by several pro-adipogenic (green) and anti-adipogenic (orange) factors. We hypothesize that Zfp68 is a potential negative regulator of adipogenesis.

Figure 1

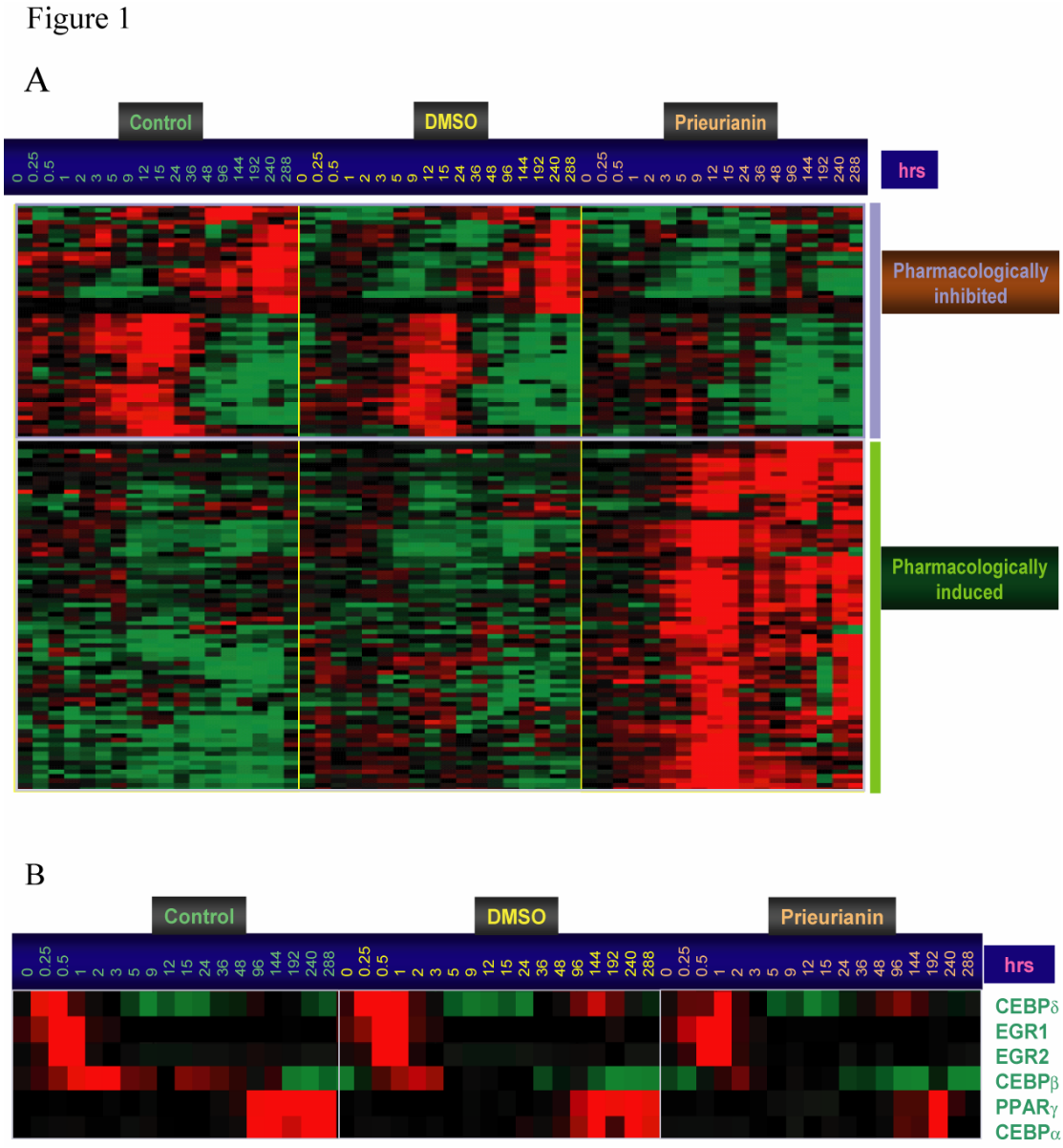
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Figure 1

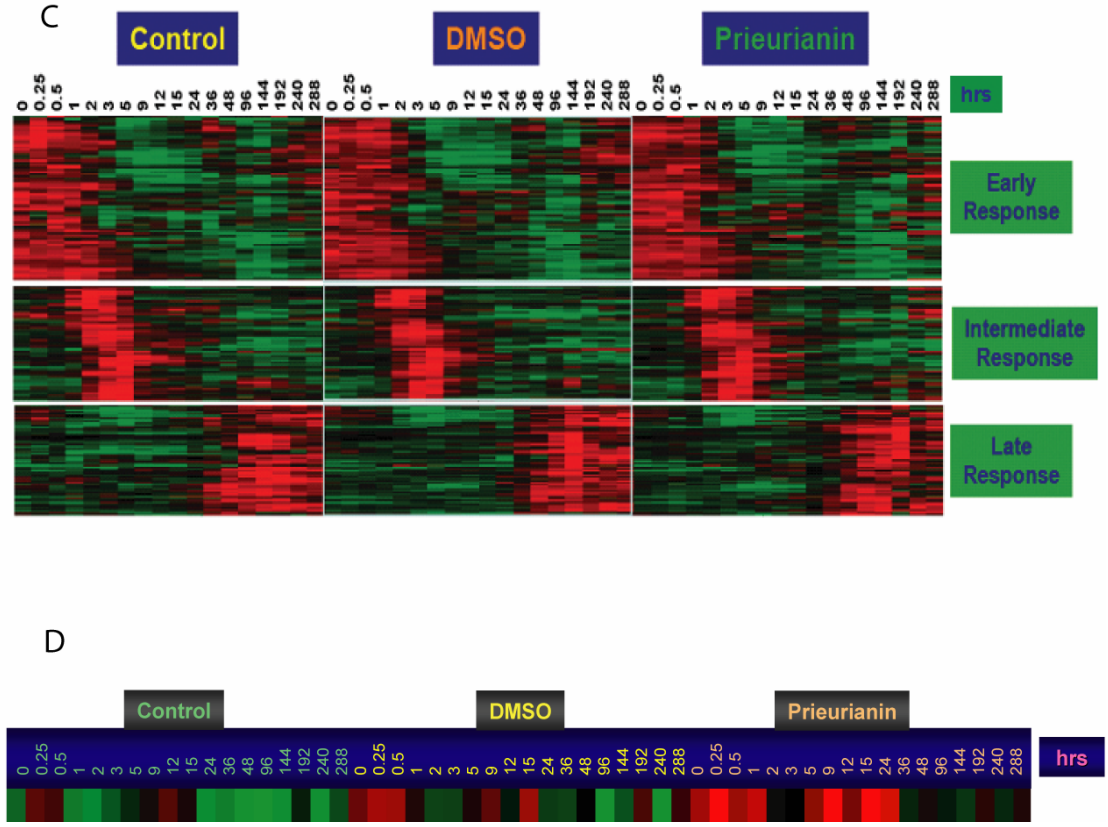
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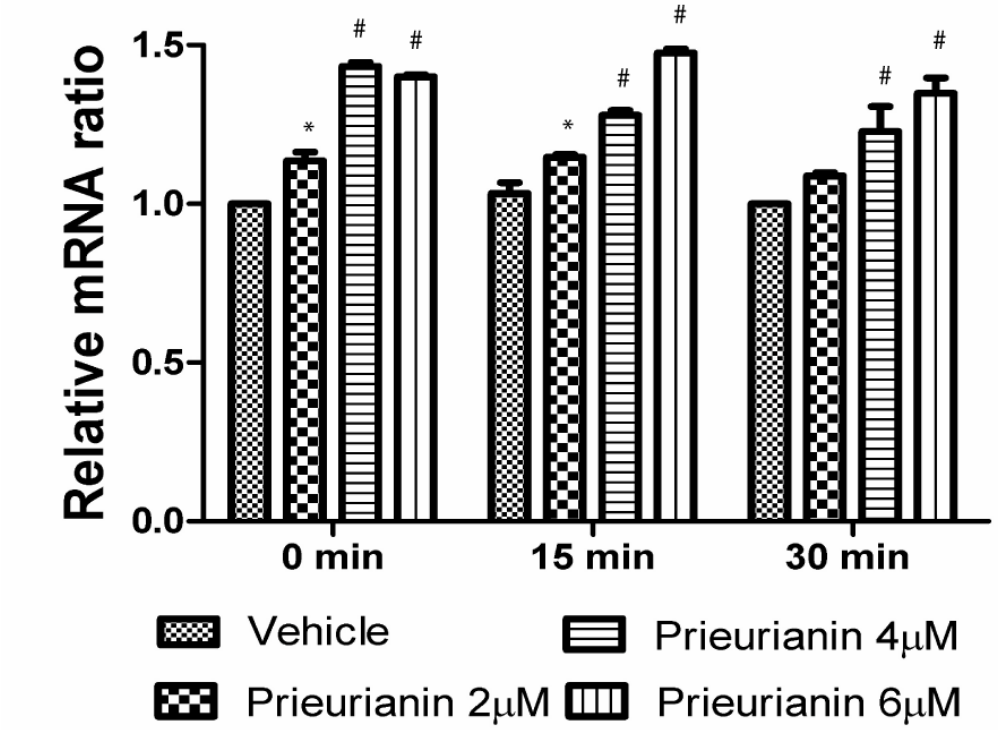
Figure 2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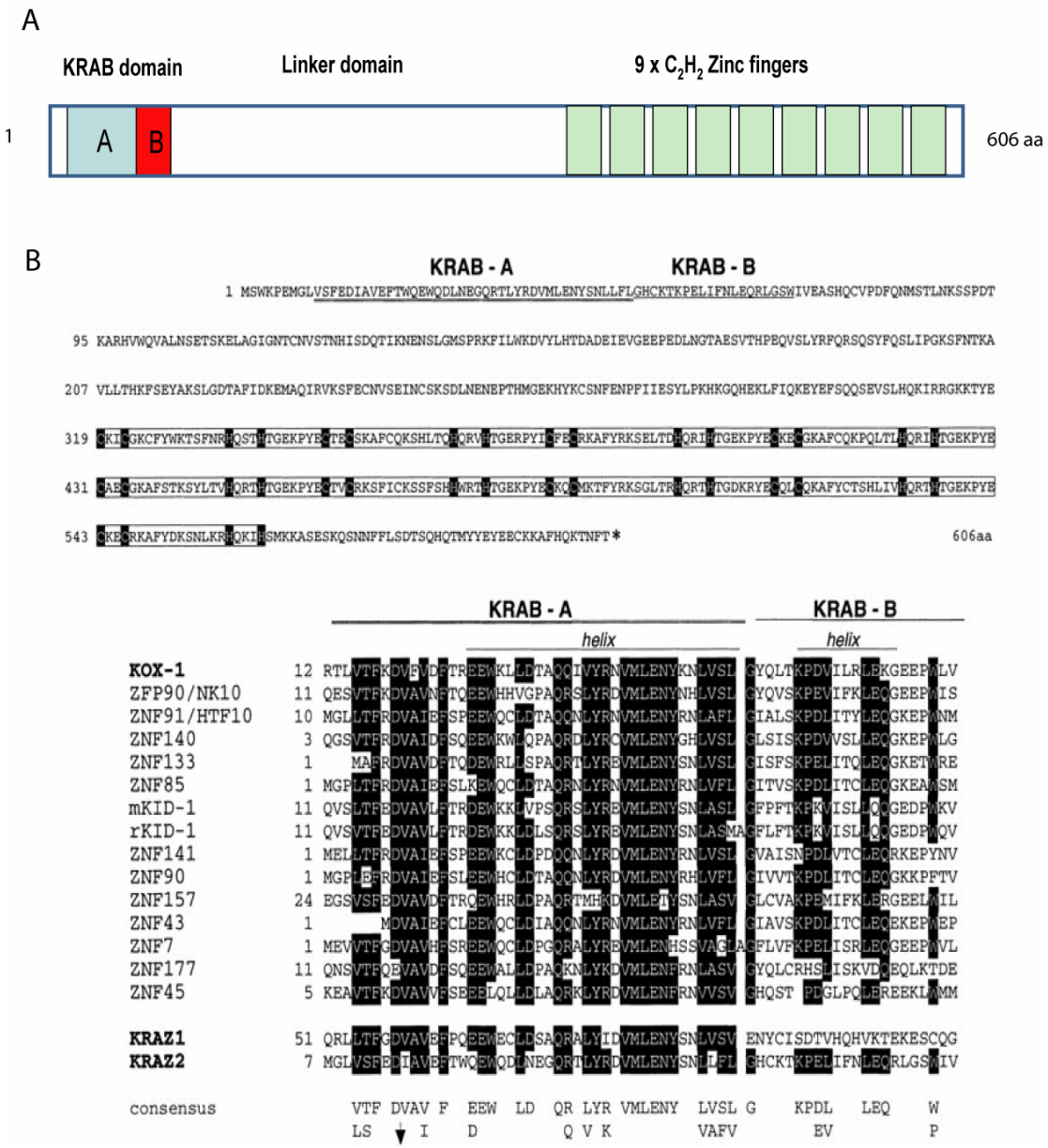
Prieurianin 6μM

Figure 3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606aa
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Figure 4

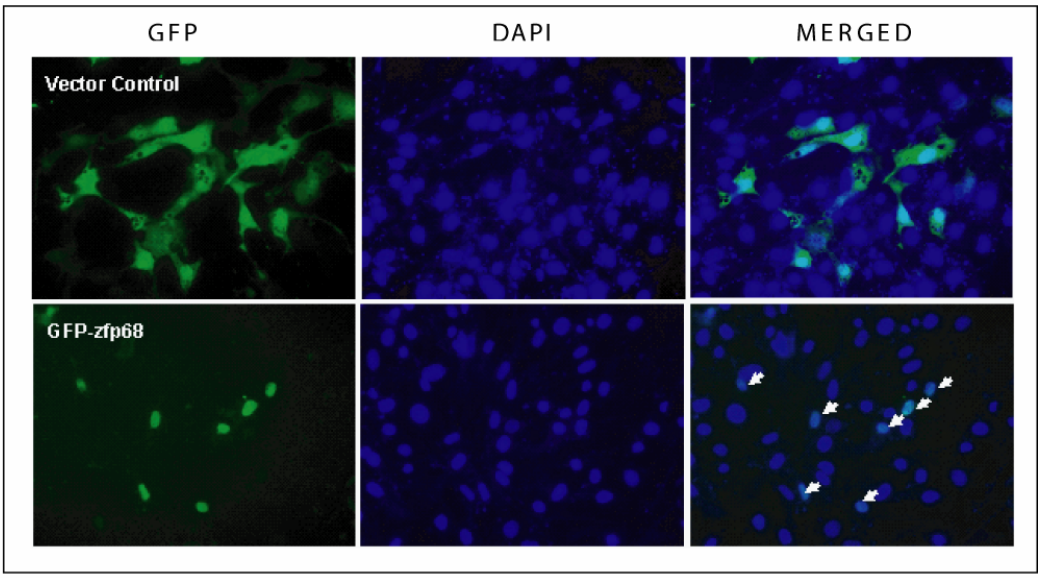
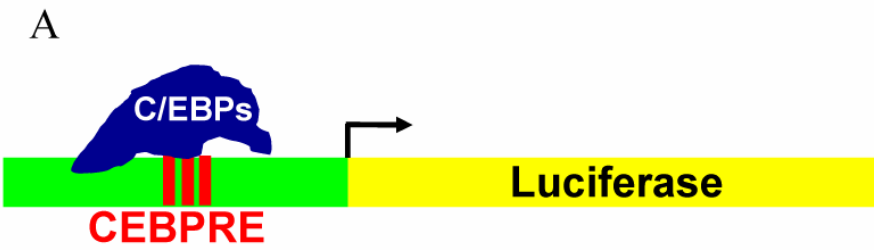
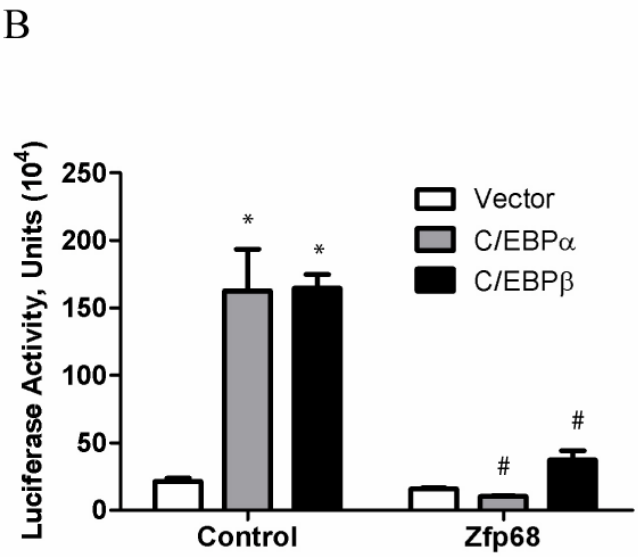


Figure 5


A
C/EBPs
Luciferase
CEBPRE


B
Luciferase Activity, Units (10^4)
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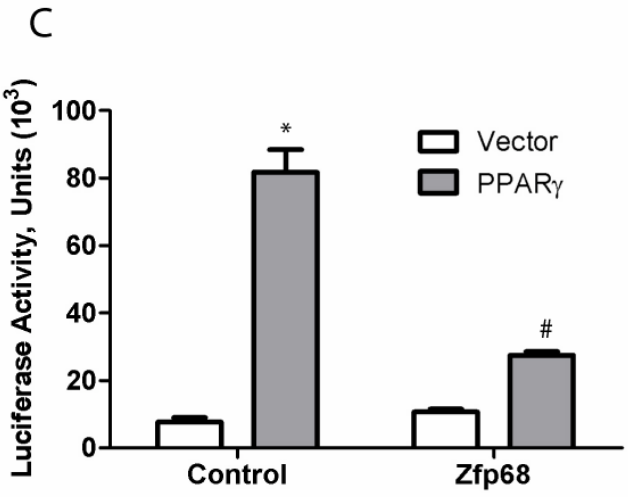
200
150
100
50
0
*
*
#
#
Vector
C/EBPα
C/EBPβ
Control
Zfp68


C
Luciferase Activity, Units (10^3)
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80
60
40
20
0
*
#
Vector
PPARγ
Control
Zfp68

Figure 6

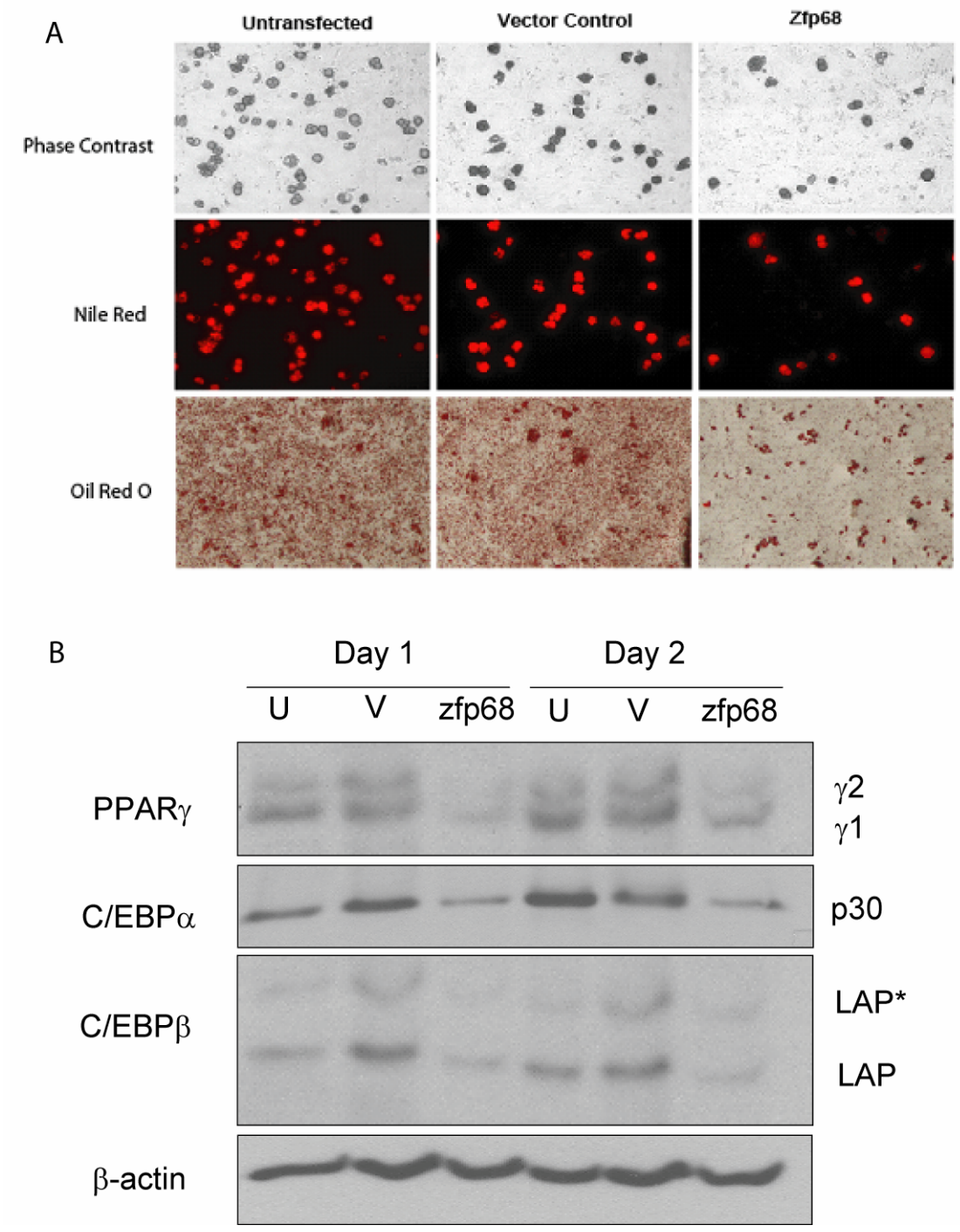
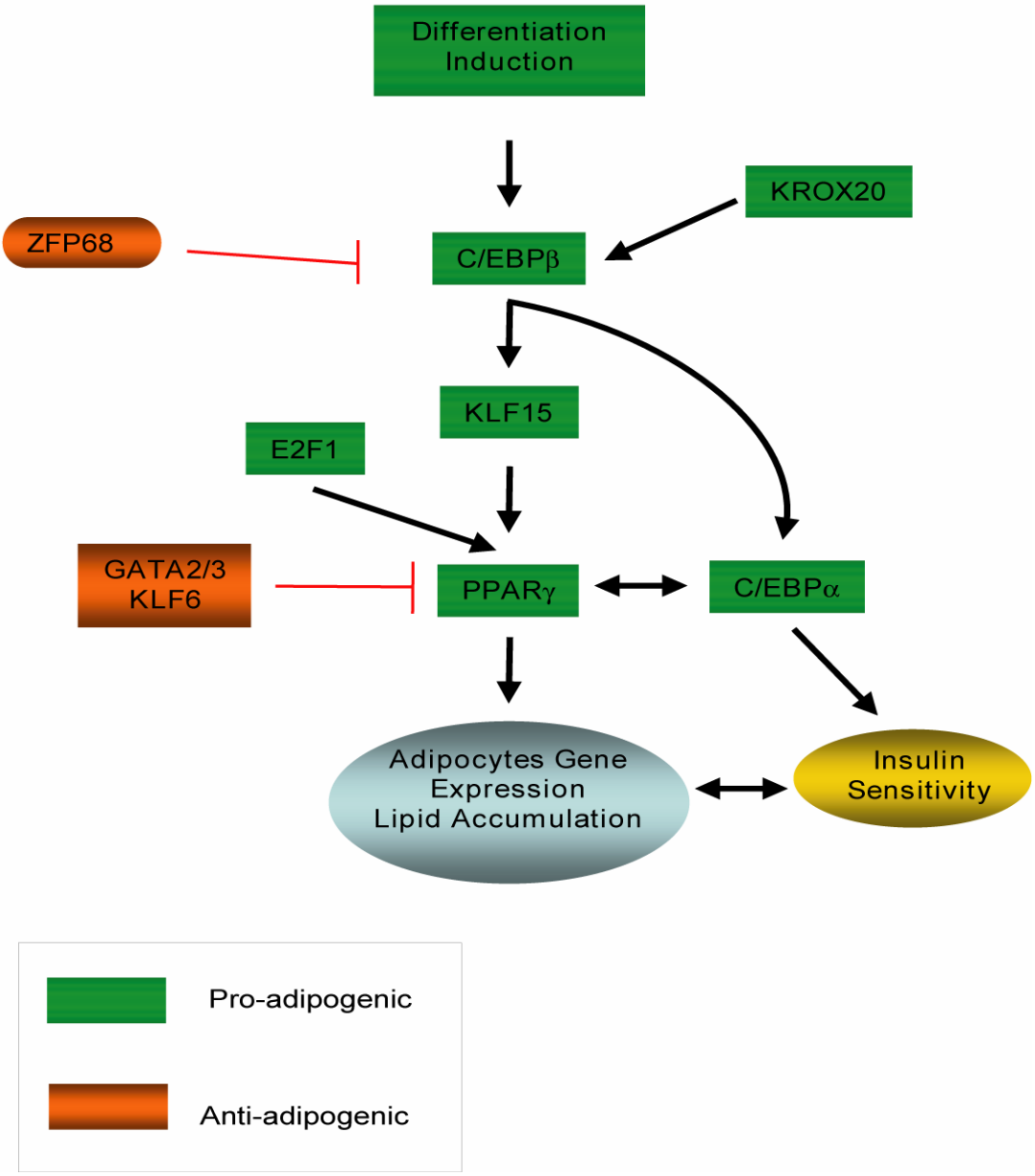

A
Untransfected
Vector Control
Zfp68
Phase Contrast
Nile Red
Oil Red O
B
Day 1
Day 2
U
V
zfp68
U
V
zfp68
PPARγ
γ2
γ1
C/EBPα
p30
C/EBPβ
LAP*
LAP
β-actin

Figure 7


Differentiation Induction
KROX20
ZFP68
C/EBPβ
KLF15
E2F1
GATA2/3 KLF6
PPARγ
C/EBPα
Adipocytes Gene Expression Lipid Accumulation
Insulin Sensitivity
Pro-adipogenic
Anti-adipogenic

Chapter IV: Manuscript 3

INHIBITION OF ADIPOGENESIS BY SALINOMYCIN*

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Running title: Salinomycin inhibits adipogenesis

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ABSTRACT

Despite intense investigation, the underlying etiologic and mechanistic causes of obesity are still not clearly understood. There is good evidence to support that overconsumption of calories from various dietary foods leads to energy imbalance, which affects the complex homeostatic system that regulates appetite and energy metabolism. However, the possibility that some particular macronutrients or bioactive ingredients in foodstuffs, which may directly disrupt the pathways that regulate energy homeostasis and cause obesity, is controversial and has not been fully addressed. The polyether ionophoric antibiotics including monensin, salinomycin, and narasin, are widely used in veterinary medicine and as food additives and growth promoters in animal husbandry including poultry farming. Their effects on human health, however, are not fully understood. Poultry consumption has risen sharply in the last three decades; therefore, we examined in this report the effects of the ionophore antibiotics on adipogenesis. Our results showed that these antibiotics inhibit the differentiation of preadipocytes into adipocytes. In addition, salinomycin, the most potent inhibitor of preadipocyte differentiation, also suppresses the transcriptional activity of CCAAT/ enhancer binding proteins. These results suggest that ionophore antibiotics or other small molecules exhibiting similar anti-adipogenic potential can be further exploited as novel anti-obesity therapeutics and as pharmacological probes for the study of adipose biology.

INTRODUCTION

Antimicrobials are used extensively in livestock farming as growth promoters and to improve health and welfare of animals by controlling coccidiosis and decreasing the shedding of zoonotic pathogens (Butaye, et al., 2003; Sapkota, et al., 2007). This widespread use of antibiotics administered at subtherapeutic levels in feed and water to promote growth and improve feed efficiency raises public health concerns with the emergence of antibiotic-resistant pathogenic strains of bacteria. Ionophoric antibiotics including monensin, salinomycin and narasin, are not used in human therapy and thus not considered medically important, therefore, they are widely used as growth promoters in animal husbandry. However, the potential human health impacts associated with the use of these antimicrobials in animal feed have not been fully examined.

Adipose tissues are increasingly recognized as a major endocrine organ that produces a diverse cadre of adipokines including leptin and adiponectin, which are involved in the modulation of energy metabolism as well as inflammatory response and the immune system (Galic, Oakhill, & Steinberg, 2009). Therefore, disruption of the homeostasis or differentiation status of adipose tissues is expected to have significant consequences on the function of this endocrine system and immune regulation. Obesity, characterized by an increase in adipose mass, is associated with increased energy intake (Swinburn, Sacks, & Ravussin, 2009), which ultimately triggers inflammation in adipose tissue (Maury & Brichard). It is postulated that chronic inflammation associated with obesity either activates or inhibits the

production of adipokines, probably through increased levels of proinflammatory cytokines. Moreover, the etiologic causes of the rise in the incidence of obesity in the past two decades and the potential link to inflammation remain unknown.

We have analyzed the Food Availability data from the USDA Economic Research Service, and observed that poultry consumption in the United States has close to doubled, while the consumption of red meat (beef, veal, lamb, and pork) has declined from the period of 1980-2007 (Figure 1). This increase in poultry consumption and the use of ionophore antibiotics in poultry and other animal farming practices, raises the question about the potential impact of consuming meat tainted with growth-promoting antibiotics on human health and prompted us to ask whether these antibiotics may have biological effects on adipose tissues. Using the cultured preadipocytes adipogenesis assays (Green & Meuth, 1974; Wolins, et al., 2006), we examined in this report the effects of the ionophore antibiotics on the differentiation of 3T3-L1 (L1) and OP9 mouse stromal cells. Our results showed that ionophore antibiotics are inhibitors of preadipocytes differentiation into adipocytes, with salinomycin being the most potent inhibitor. Further analysis showed that salinomycin also inhibited the transactivation potential of the CCAAT/enhancer binding proteins (C/EBPs), which are major transcriptional regulators of adipogenesis. Salinomycin has recently been shown to be a specific inhibitor of cancer stem cells (P. B. Gupta, et al., 2009). This, coupled with our findings, suggest the potential development and application of ionophore antibiotics or small molecule derivatives possessing similar anti-adipogenic activity as novel therapeutics for combating obesity. Further, the unique biological effects of these agents on the function of stem cells and the differentiation of preadipocytes suggest their use as a pharmacological tool for probing and understanding adipose and stem cell biology.

MATERIAL AND METHODS

Cell culture, chemicals

Bone marrow-derived mouse stromal OP9 cells (Wolins, et al., 2006) (a gift of Dr. Perry Bickel, University of Texas Health Science Centre, Houston) were cultured at 37°C in OP9 propagation media: MEM-α with 20% FBS (Premium Fetal Bovine Serum, catalog no. 14-051Q, Atlanta Biologics), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. 3T3-L1 (L1) cells, obtained from the American Type Culture Collection were cultured in 3T3-L1 propagation media: DMEM with 10% bovine serum (Invitrogen, catalog no. 16170-078), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Ionophore antibiotics were obtained from Sigma and dissolved according to manufacturer's recommendation.

Differentiation

OP9 were grown to confluence and then cultured for 2 additional days in OP9 propagation media. The cells were then cultured up to 3 more days in serum replacement (SR) media: MEM-α (HyClone, SH 3026501) with 15% KnockOut Serum Replacement (Invitrogen, catalog no. 10828-028), 100 U/ml penicillin, and 100 µg/ml streptomycin, and then replenished in the propagation medium.

3T3-L1 cells were differentiated as previously described (Student, et al., 1980). In brief, the L1 cells were grown to confluence and then cultured for 2 additional days in 3T3-L1 propagation media. Cells were then cultured 2 days in DM1: DMEM with

10% FBS, 175 nM insulin, 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were then replenished with DM2: DMEM with 10% FBS, 175 nM insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. At that point, 3T3-L1 cells were maintained in 3T3-L1 adipocyte media. Lipid accumulation in adipocytes was visualized by staining with Oil Red-O or Nile Red (Sigma).

Plasmid, Transfection and Reporter Assays

C/EBP response-elements reporter plasmids were constructed by ligating oligonucleotides containing either three C/EBP-response elements in tandem into the pGL3-Luc reporter expression vector and sequence-verified. The mouse PPARγ_2 gene promoter was amplified as previously described (Zhu, et al., 1995) with primers: forward (5'-CTAGCTAGCGCTCCCAC-GTTAGCAGTTTGGCAC-3'and reverse 5'-CCCAAGCTTCTTGCAGCAACATCAGGAATGGAC-3') including *Nhe* I and *Hind* III sites, subcloned into pGL3-basic vector (Promega) and sequence verified.

The melanoma Mel501 cells (American Type Culture Collection) or OP9 cells were transfected with expression constructs for C/EBPs, and PPARγ and contransfected with zfp68 expression construct using the Lipofectamine 2000 transfection reagent (Invitrogen). Equal amounts of DNA were used for all transfection by adding the appropriate amount of salmon sperm DNA and the *Renilla* luciferase reporter as internal control. Relative luciferase activities were determined 24 h following transfection and normalized to the *Renilla* luciferase activity. All transfection experiments were performed in triplicates.

In vitro transcription

A HeLa scribe Nuclear Extract in vitro transcription (Promega Cat. No. E3110) was used for in vitro transcription with T7 linerized Luciferase DNA Control (Promega Cat. No P141A). The reaction mixture was incubated with the drug for 10 min at RT prior to adding the T7 Polymerase to initiate the reaction. Then the mixture was incubated at 30ºC for 60 min. RNA was purified using RNA Isolation Kit (Qiagen Cat. No. 74104) and eluted in 35 µl of Nuclease Free Water. 5µl of the RNA was mix with an equal volume of Loading Dye, incubated for 10 min at 95ºC prior to loading onto a gel and cool down on ice. The samples were run on 5% TBE-urea gel (Bio-Rad cat. No. 1611115) and the gel was exposed to X-ray film for 10 min at RT.

In vitro translation

A Wheat Germ Extract (Promega Cat No. L4380) was employed for *in vitro* translation. Reactions programmed with 1 µg of luciferase RNA ± drugs were incubated at 25ºC for 120 min and 2 µl of cell lysate was mix with 45 µl of the substrate and read on luminometer.

Protein Synthesis - ^{35}S Methionine incorporation assay

For in labeling experiments, NIH 3T3-L1 cells were plated in 24 well plates at a density of $5x10^4$ cells/well. 1 hour before labeling began; exponentially growing cells were treated with or without the drug for 1 hr. After 1 hr the cells were labeled with ^{35}S Methionine (50µCi/ml) in methionie free medium (Sigma, DMEM Cat. No. D0422 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin) ± drug for an additional 30 minutes, to ensure optimal incorporation of ^{35}S Methionine. Cells were washed 1x with ice cold PBS and

precipitated with 10% TCA. The plate was spun down at 1000 x rpm and the solution was aspirated. 100ul of 5 M NaOH was added to dissolve the protein and added into 3 ml of scintillation liquid.

Cell viability

Viability of cell populations in culture was quantified by metabolic activity assay, measuring the conversion of 3- [4,5-dimethylthiazol2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to the formazan derivative using the Cell Growth Determination kit from Sigma (St. Louis, MO). The formazan derivative was quantified by measuring its absorbance at 570 nm with a Spectra Max 384 Plus plate reader.

Annexin V Assay

Attached cells were harvested by trypsinization and pooled with any detached cells in the medium. The cells were then stained with the Guava Nexin reagent (Guava Technologies, Hayward, CA, USA), containing pre-mixed annexin V-PE and 7-aminoactinomycin D (7-AAD), following the manufacturer's protocol. Annexin V positive cells were quantified by flow cytometry, using a Guava Personal Cytometer.

Statistical analyses

Commercially available software package (PRISM, GraphPad Software, Inc., La Jolla, CA) or the data analysis add-in for Microsoft Excel was used for performing statistical analysis. Either single factor ANOVA or Student's t-tests was used for all analysis. P values of less than or equal to 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The ionophoric antibiotics are a group of polyether alkali monovalent cationic transporter with monensin exhibiting selectivity for Na^+, and salinomycin and narasin (4-methylsalinomycin) showing preference for K^+ over Na^+ (Figure 2) (Riddell, 2002; Samanidou & Evaggelopoulou, 2008). We assessed the biological activity of these antibiotics on adipogenesis using cultured OP9 stromal cells. 3T3-L1 preadipocytes and OP9 stromal cells were induced to differentiate into adipocytes either in the absence or in the presence of the antibiotics. Our results showed that Salinomycin and monensin inhibited the differentiation of OP9 cells with salinomycin showing a greater potency than monensin (Figure 3b), compared to TNFα, which is known to inhibit the differentiation of preadipocytes into mature adipocytes (Figure 3a) (Simons, van den Pangaart, van Roomen, Aerts, & Boon, 2005). Greater than 90% inhibition of preadipocytes differentiation was observed with either 10 nM salinomycin or 50 nM monensin (Figure 3b). In contrast, narasin, a derivative of salinomycin showed little inhibition of differentiation at 100 nM (Figure 3b).

It is noteworthy that under the culture condition of the adipogenesis assay, inhibition of differentiation by salinomycin in OP9 cells was not a result of drug induced apoptosis as indicated by the lack of annexin V binding to phosphatidylserine, compared to cells treated with doxorubicin, a cytotoxic drug that causes cell death (Figure 4a and b). Both monensin (Na^+-selective) and salinomycin (K^+-selective) inhibited differentiation. Salinomycin was the most potent inhibitor among the group; therefore, we used it as the prototype for all ensuing studies.

Rosiglitazone, a member of the thiazolidinedione class of anti-diabetic drugs, is an activator of PPARγ and known to induced adipogenesis (Sharma & Staels, 2007). We differentiated OP9 cells with rosiglitazone either in the absence or in the presence of salinomycin. Our results showed that salinomycin blocked rosiglitazone-induced adipogenesis (Figure 5a and b), thus suggesting that salinomycin might block the transcriptional regulation of adipogenesis mediated by PPARγ.

The transcription factors C/EBPα, β, and δ, and PPARγ are considered master regulators of adipogenesis (Lefterova & Lazar, 2009). Since salinomycin blocks rosiglitazone-induced adipogenesis, we asked whether salinomycin might pharmacologically repress the transactivation potential of C/EBPs, using promoter reporter assay driven by the C/EBP response elements. Our results showed that salinomycin inhibited C/EBPα-, C/EBP β-, C/EBPδ–mediated transactivation from their respective response element driven reporters (Figure 5c, d and e). In addition, C/EBPs induced transactivation from the PPARγ native gene promoter was also inhibited by salinomycin (Figure 5f).

That salinomycin inhibits C/EBPs activated gene expression, points to its pharmacological potential as a transcription inhibitor. To determine whether salinomycin is a transcription inhibitor, we examined its effects on cell-free HeLa nuclear extracts driven *in vitro* transcription, and our results showed that salinomycin did not inhibit transcription from a synthetic DNA template (Fig. 6a). Furthermore, salinomycin also did not inhibit protein synthesis neither from a luciferase transcript in a wheat germ cell-free extract or in ^{35}S-methionine incorporation assay (Figure 6b and c).

Salinomycin was recently shown to be a selective and potent cytotoxic agent against mammary cancer stem cells (P. B. Gupta, et al., 2009). Therefore, we further

investigated the cytotoxic effects of salinomycin in OP9 and L1 cells. Cell count and MTT assays showed that OP9 cells (Figure 7a and b) were more sensitive to the cytotoxic effects of salinomycin than L1 cells (Figure 7c and d).

The widespread use of ionophore antibiotics in animal husbandry and the concerns for such practice on human health prompted us to investigate the biological effects of these antimicrobials on adipogenesis. Our results revealed unexpectedly that these agents are potent inhibitors of preadipocytes differentiation into adipocytes, probably via their repression of the transcriptional activity of the master regulators of adipogenesis including C/EBPα, β, and δ, and PPARγ.

Salinomycin was recently shown in a high-throughput screen study to have selective and potent cytotoxicity against cancer stem cells (P. B. Gupta, et al., 2009). Salinomycin has also been shown to inhibit mitochondrial oxidative phosphorylation, causing the release of K^+ from mitochondria (Mitani, Yamanishi, Miyazaki, & Otake, 1976). At concentrations up to 100-fold higher than those used in our study, salinomycin has also been shown to induce apoptosis (Fuchs, Heinold, Opelz, Daniel, & Naujokat, 2009). These observations together with the results from our study offer further insights into the unique pharmacological profiles of these ionophore antibiotics.

Is the block of differentiation a result of the cytotoxic effects of salinomycin that leads to drug-induced onset of apoptosis? It is possible that differentiation inhibition by salinomycin can be attributed to its cytotoxic effects, which is supported in part by the observation that OP9 cells (Figure 7 a and b) are more sensitive to salinomycin than 3T3-L1 cells (Figure 7 c and d). We reason that the differential sensitivity to salinomycin is due to the presence of more stem or stem-like cells in the bone marrow derived OP9 mouse stromal cells than the embryonic fibroblast L1 cells (Green &

Meuth, 1974; Wolins, et al., 2006). Moreover, OP9 cells have been demonstrated to differentiate more readily and with greater robust than 3T3-L1 cells. Therefore, the inherently larger subset of stem or stem-like cells within the population OP9 cells in culture, are selectively targeted by salinomycin, thus accounting for the observed differentiation sensitivity between OP9 and 3T3-L1 cells.

The premise that differentiation inhibition is a result of the cytotoxic effect of salinomycin is plausible, but is eclipsed by the lack of annexin V positive cells in the salinomycin treated OP9 cells, hence suggesting that salinomycin-inhibited OP9 cells differentiation may not be attributed to its cytotoxicity alone. Since salinomycin also inhibits the transcriptional activity of C/EBPs and PPARγ, these results, therefore, raise the possibility that other cellular mechanism might be involved in the block of differentiation. The specific inhibition of the transactivation of PPARγ and C/EBPs also suggests that the mechanism of transcriptional repression can be mediated by post-translational protein modification including phosphorylation.

Although either Na^+ or K^+ may be required for their action, the ability of both monensin and salinomycin in inhibiting preadipocytes differentiation into adipocytes further suggests that the mechanism of inhibition of adipogenesis is probably independent of the presence of a specific alkali cation. These results suggest there might be additional molecular targets that mediate the inhibition of adipogenesis by these antimicrobials.

The muted biological activity of narasin in inhibiting preadipocytes differentiation points to a potential pharmacophore for these polyether antibiotics as anti-adipogenic compounds. Narasin (4-methylsalinomycin) is a derivative of salinomycin with an additional methyl group on the terminal tetrahydropyrane ring (Figure 2). We speculate that the core structure that is essential for the anti-adipogenic activity of

salinomycin and monensin probably encompasses the tetrahydropyrane ring tethered to the monocarboxylic acid. The medicinal chemistry of salinomycin will undoubtedly reveal the pharmacophore of these molecules. Whether the anti-adipogenic potential and the anti-cancer stem cell biological activity are overlapping remains to be determined.

In sum, our results demonstrated the anti-adipogenic activity of some of the ionophore antibiotics on preadipocytes. These compounds, particularly salinomycin, also inhibit the transcriptional activity of the master regulators of adipogenesis. These compounds can be further developed as novel anti-obesity therapeutics and as pharmacological tools for probing the biology of adipose tissues.

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FIGURE LEGENDS

Figure 1. Consumption of poultry vs. consumption of red meat in years 1980-2007. (Food Availability data from the USDA Economic Research Service). USDA data shows, that beef consumption has trended downward since 1980s while pork consumption has remained fairly stable over the last two decades. Per capita poultry consumption has grown consistently over the last 40 years and now exceeds beef and pork consumption

Figure 2. Structures of three polyether ionophore antibiotics: monensin, narasin and salinomycin. Monensin, narasin and salinomycin are ionophore antibiotics produced by *Streptomyces,* with five or six membered ether rings and hydroxyl groups at one terminus, and a carboxyl moiety at the other. The "polyether" nature of the ionophore refers to the considerable number of tetrahydro-pyrans and –furans found in the ionophore structure.

Figure 3. Anti-adipogenic effect of ionophore antibiotics in OP9 and 3T3-L1 cells. Adipocyte differentiation was observed by phase contrast microscopy (left panel) and Nile Red fluorescence staining (right panel). **a**, OP9 preadipocytes were induced and with a Knockout Induction Media as described previously in *Material and Methods* and treated with TNFα, a cytokine that inhibits differentiation of preadipocytes into mature adipocytes is used as positive control, compared to untreated or MeOH, vehicle control. **b,** Monensin and salinomycin inhibit OP9 adipocytes differentiation,

with salinomycin showing a grater potency then monensin as measured by staining with Nile Red **c,** Salinomycin inhibits the differentiation of 3T3-L1 cells into adipocytes in dose dependent manner, stained with Nile Red, compared to untreated or vehicle-treated adipocytes. TNFα, which causes lipolysis, serves as positive control and Rosiglitazone promotes development of adipocytes.

Figure 4. Salinomycin does not induce apoptosis in preadipocytes undergoing differentiation **a,** OP9 cells were treated with 10, 50 and 75 nM of salinomycin for 3 days. Doxorubicin, known apoptotic agent, serves as a positive control. Attached and detached cells were pooled for flow cytometry analysis. Parallel cultures treated with MeOH served as controls. **b,** Graphical quantification of Annexin V positive cells.

Figure 5. Salinomycin attenuated rosiglitazone-induced adipocytes and inhibited transcriptional activity of C/EBPs. OP9 preadipocytes were induced to differentiate and treated with Salinomycin 10, 50 nM and/or rosiglitazone 2 μM at the same time for 3 days. **a,** Adipocyte differentiation was observed by phase contrast microscopy (left panel) and Nile Red staining (right panel) **b,** Oil Red O staining of adipocytes of single well from 24-well plate. **c,d,e,** Salinomycin inhibited C/EBPα-, C/EBP β-, C/EBPδ–mediated transactivation activation of C/EBPRE and PPARγ native gene promoter (**f**). Statistics were conducted as student t-test. Asterisk, p,<0.05 vs. vector; hash p<0.05 vs. the respective C/EBPα-, C/EBP β-, C/EBPδ -transfected controls. Results are means ± S.E.M., normalized to *Renilla* luciferase activity.

Figure 6. Salinomycin is not an inhibitor of transcription and translation. **a,** Salinomycin does not inhibit transcription in the *in vitro* transcription system.

Transcription product generated from the in vitro transcription of the HeLa Nuclear extract Luciferase Positive Control DNA. 5ul of product was subjected to electrophoresis on 5% TBE-urea gel and exposed to film. Salinomycin did not inhibit translation neither in the in vitro translation system as quantified by luminometer (**b)** or as measured by the amount of protein synthesized in the presence of ^{35}S methionine (**c**). Cyclohexamide (1μM), inhibitor of protein translation, and actinomycin D (10μM), inhibitor of transcription, were used as a corresponding control. Statistics were conducted as student t-test. Asterisk, p,<0.05 vs. control. Results are means ± S.E.M.

Figure 7. Salinomycin inhibits the proliferation of preadipocytes OP9 or 3T3-L1 cells. OP9 (**a**) and 3T3-L1 (**c**) cells were seeded in 24 well plate density 5000 cells/well, treated with salinomycin 10, 50, 100 nM in triplicates for 6 days. Each day the cells were trypsinized and count on Bekman Coulter. Data shown represents the means ± S.E.M. of triplicate experiments. OP9 (**b**) and 3T3-L1 (**d**) cells were cultured with various concentrations of Salinomycin (1-500 nM). After 2 days, cell proliferation was measured by MTT assay. Results represent the mean ± S.E.M. of experiments performed in triplicate. Asterisk, p,<0.05 vs. control.

Figure 1

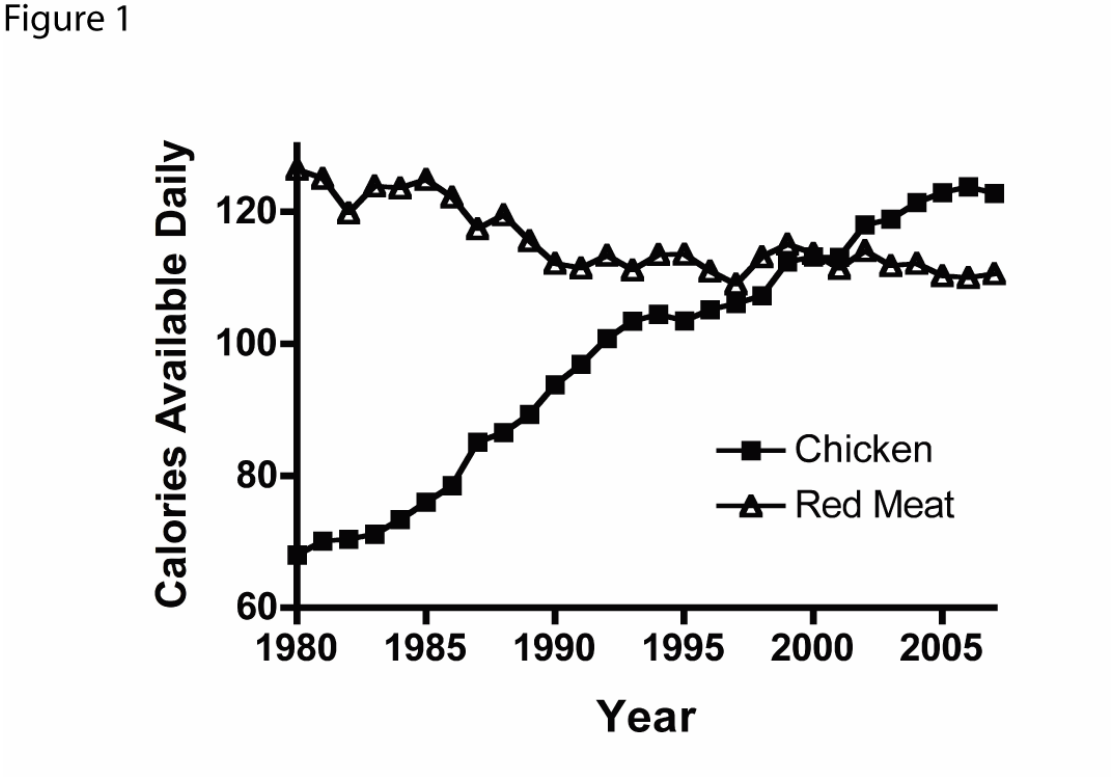

Calories Available Daily
120
100
80
60
1980
1985
1990
1995
2000
2005
Year
Chicken
Red Meat

Figure 2

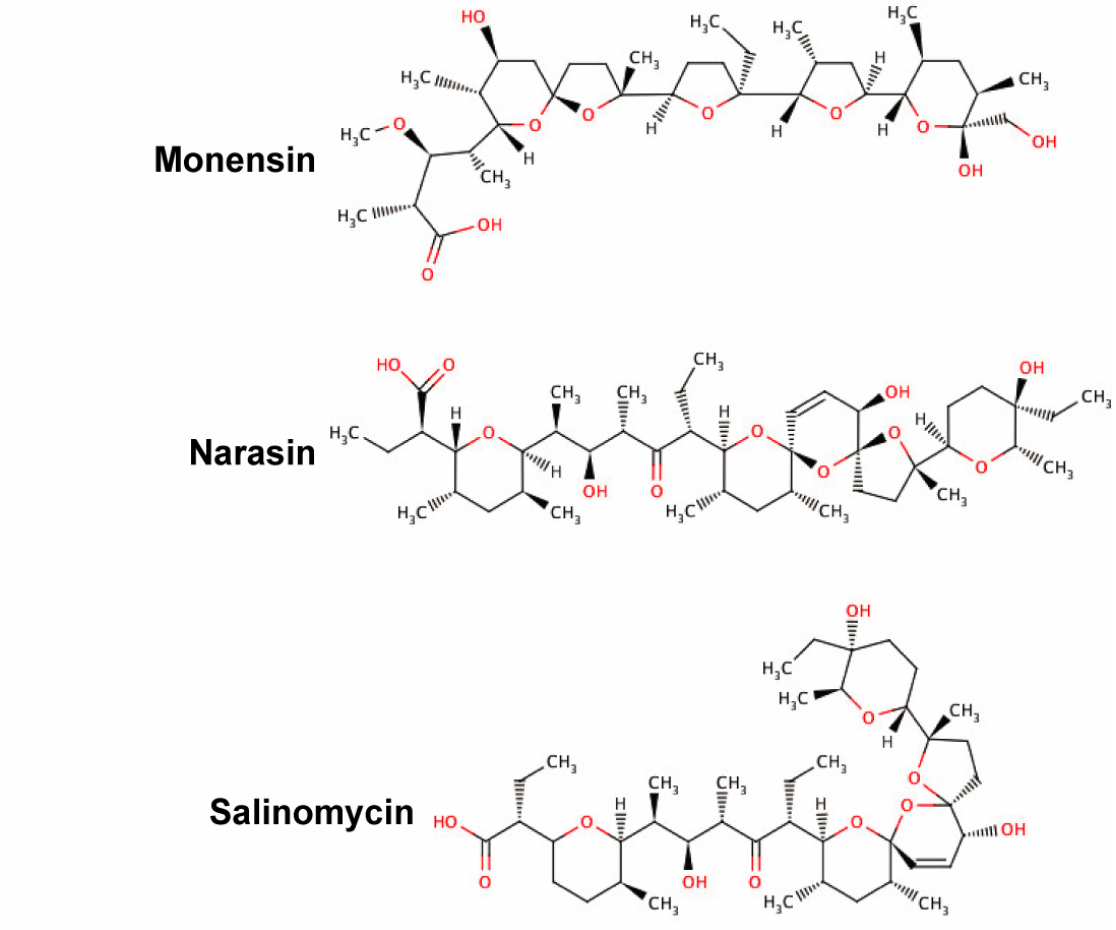


Figure 3 a

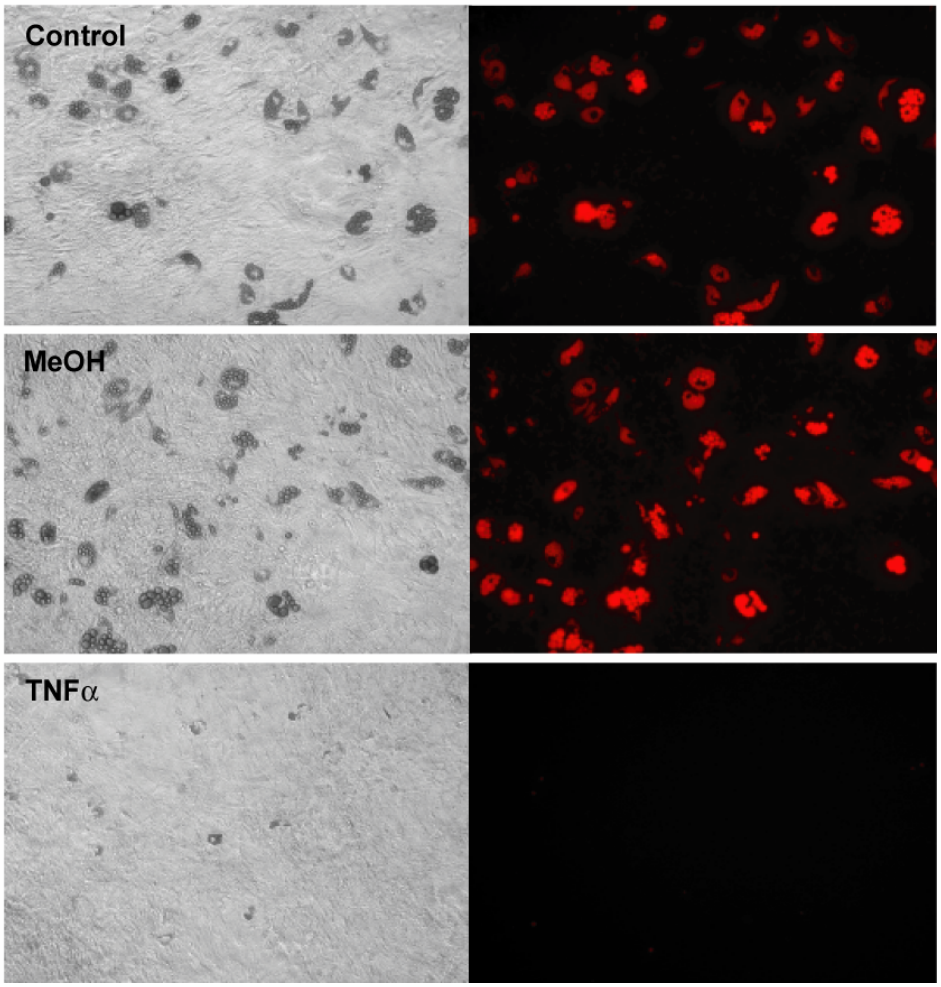


Figure 3b

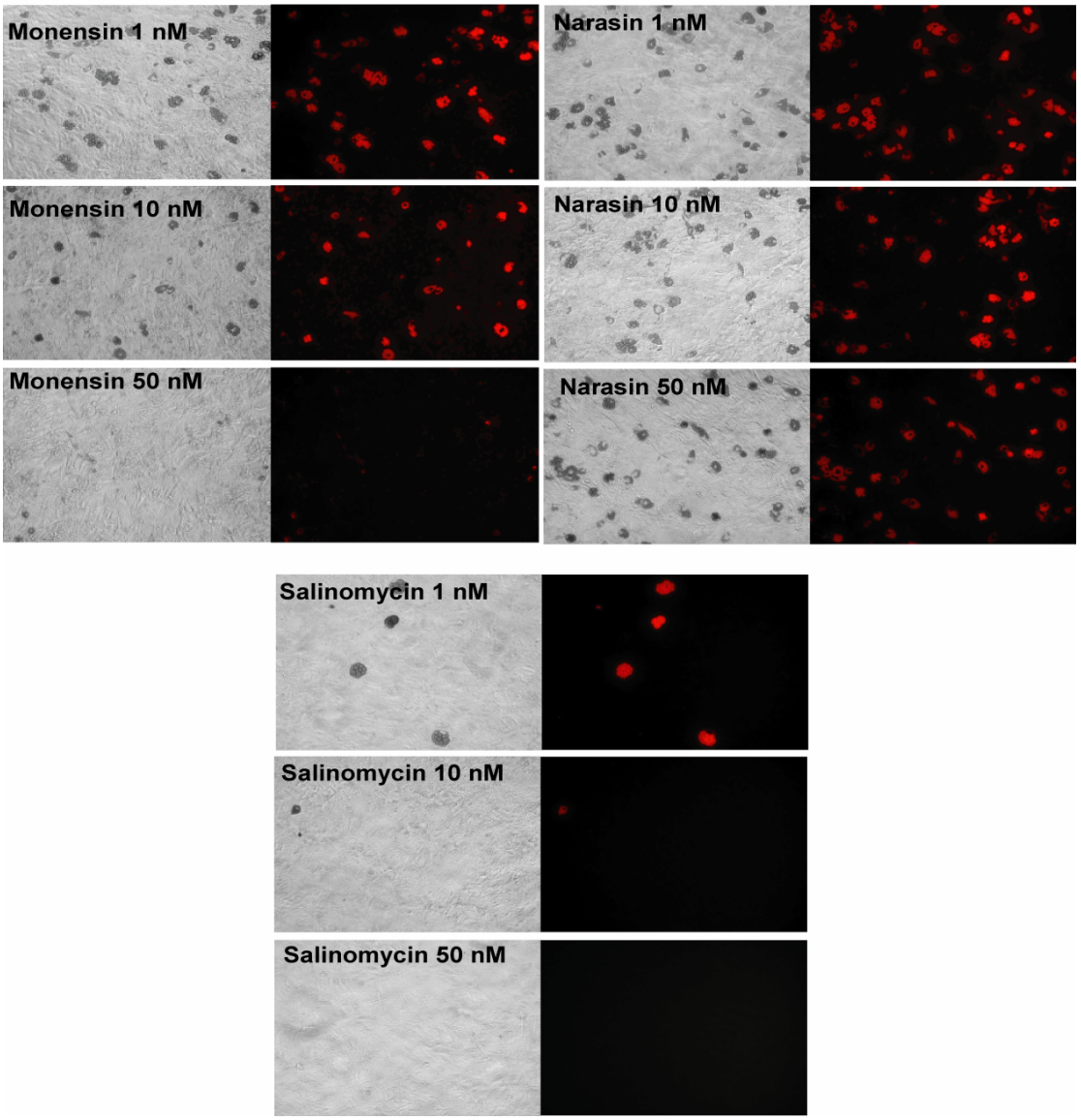

Monensin 1 nM
Narasin 1 nM
Monensin 10 nM
Narasin 10 nM
Monensin 50 nM
Narasin 50 nM
Salinomycin 1 nM
Salinomycin 10 nM
Salinomycin 50 nM

Figure 3 c

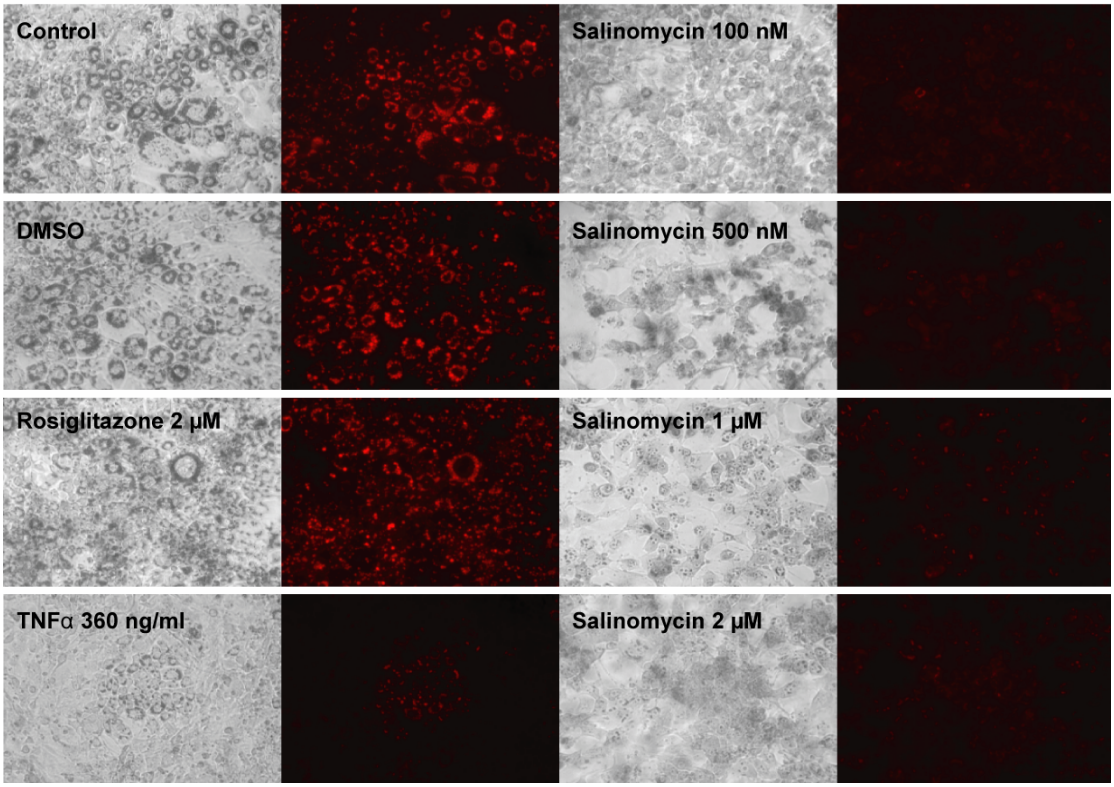
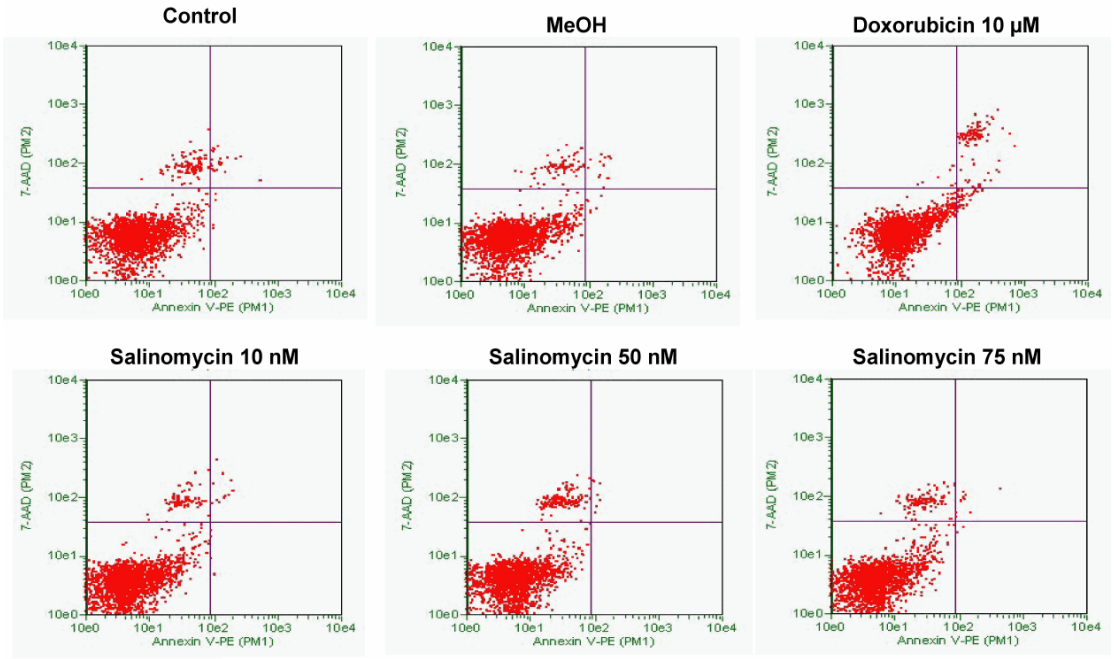

Control
Salinomycin 100 nM
DMSO
Salinomycin 500 nM
Rosiglitazone 2 µM
Salinomycin 1 µM
TNFα 360 ng/ml
Salinomycin 2 µM

Figure 4

a


Control
MeOH
Doxorubicin 10 µM
Salinomycin 10 nM
Salinomycin 50 nM
Salinomycin 75 nM
7-AAD (PM2)
Annexin V-PE (PM1)
10e0
10e1
10e2
10e3
10e4

b

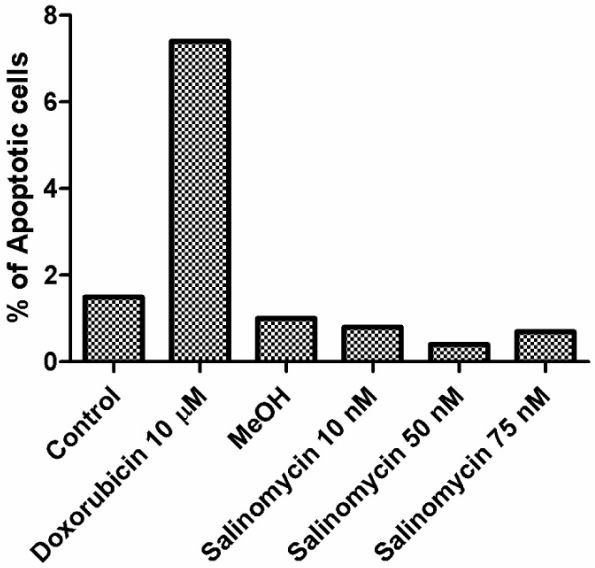

% of Apoptotic cells
0
2
4
6
8
Control
Doxorubicin 10 µM
MeOH
Salinomycin 10 nM
Salinomycin 50 nM
Salinomycin 75 nM

Figure 5

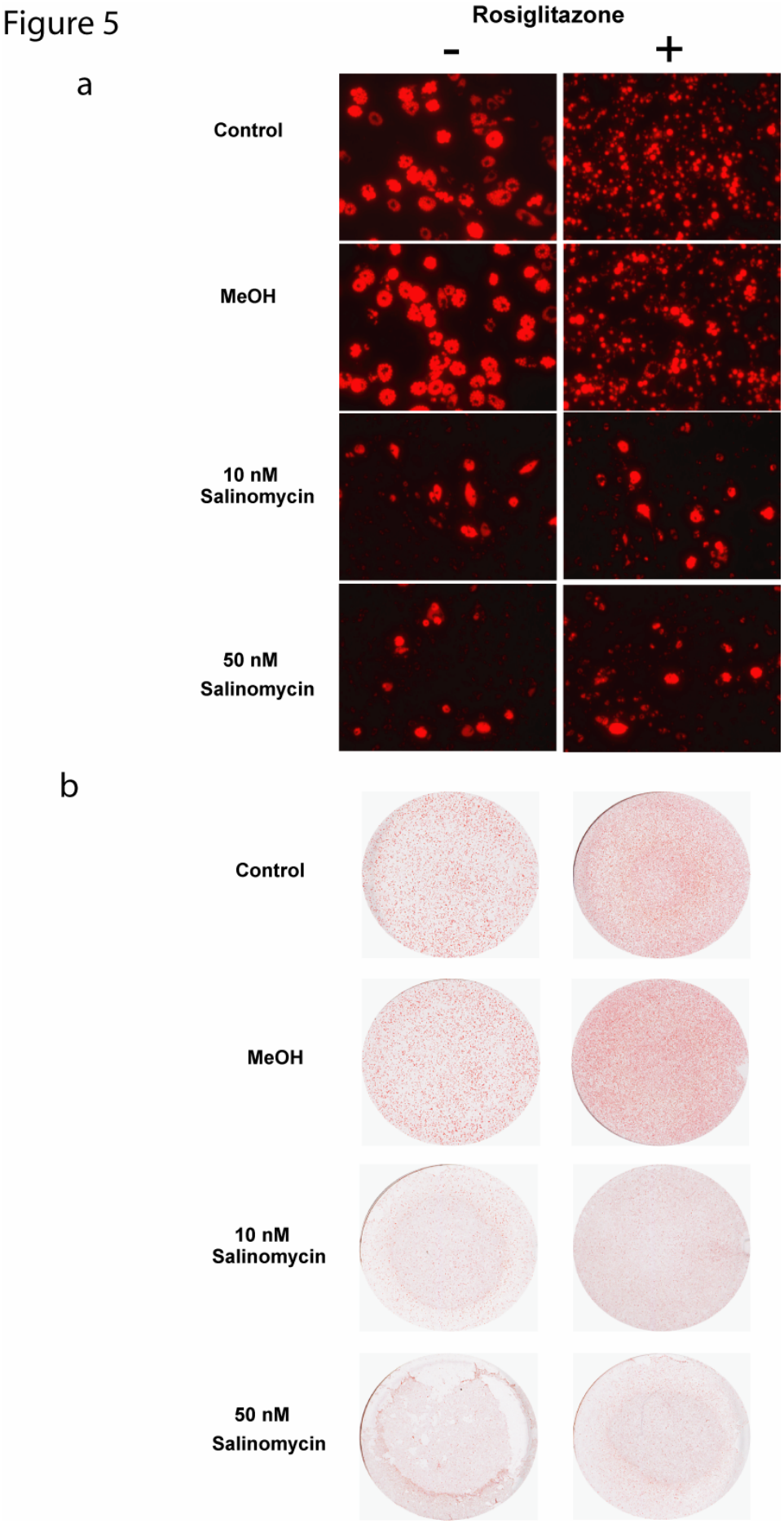
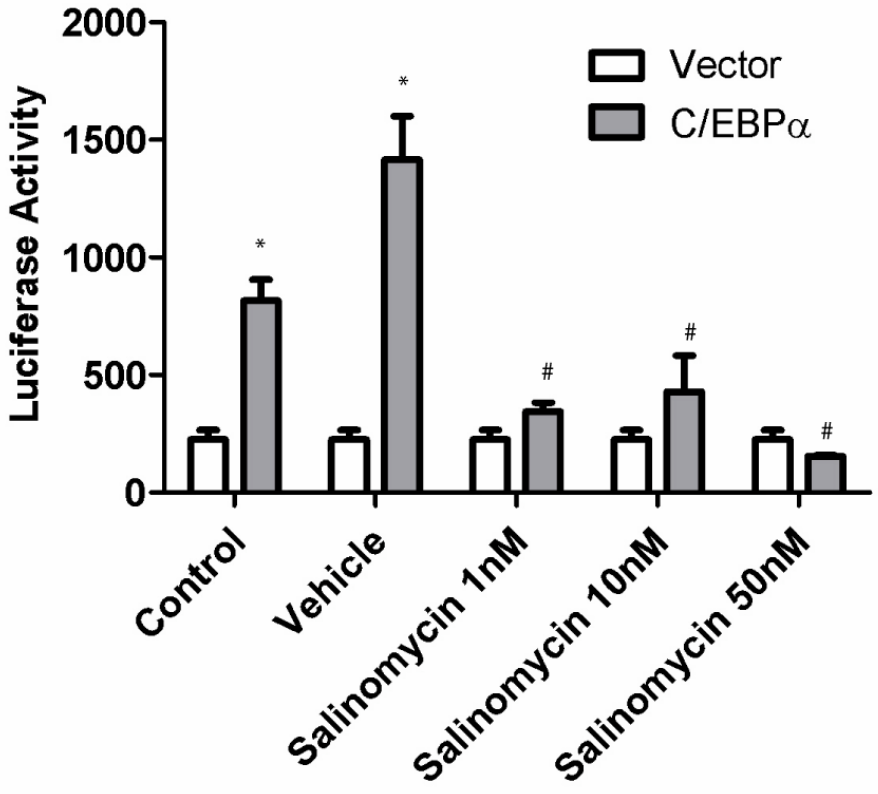

a
Rosiglitazone
-
+
Control
MeOH
10 nM
Salinomycin
50 nM
Salinomycin
b
Control
MeOH
10 nM
Salinomycin
50 nM
Salinomycin

Figure 5

c


2000
1500
1000
500
0
Luciferase Activity
Vector
C/EBPα
*
*
#
#
#
Control
Vehicle
Salinomycin 1nM
Salinomycin 10nM
Salinomycin 50nM

d

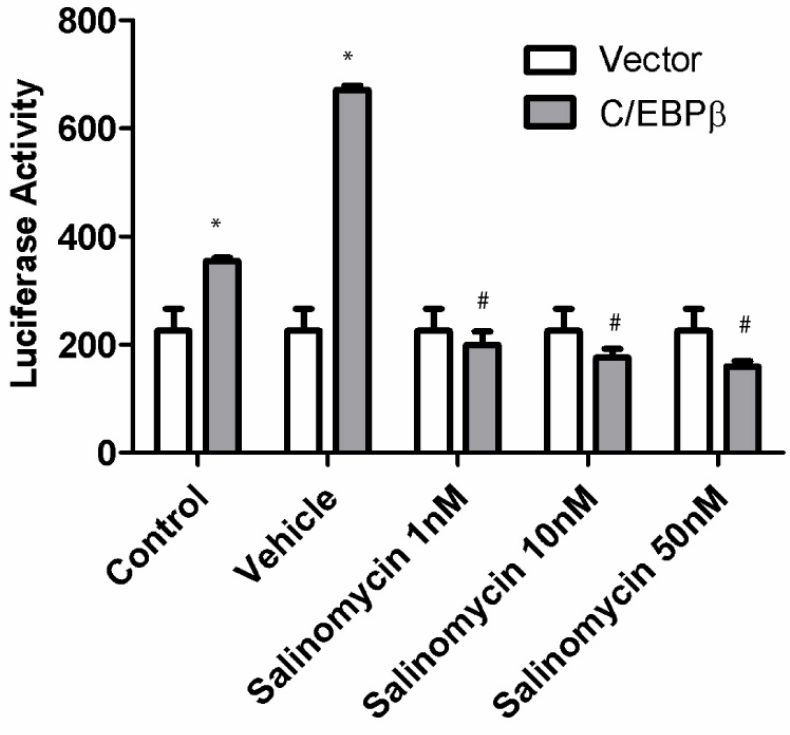
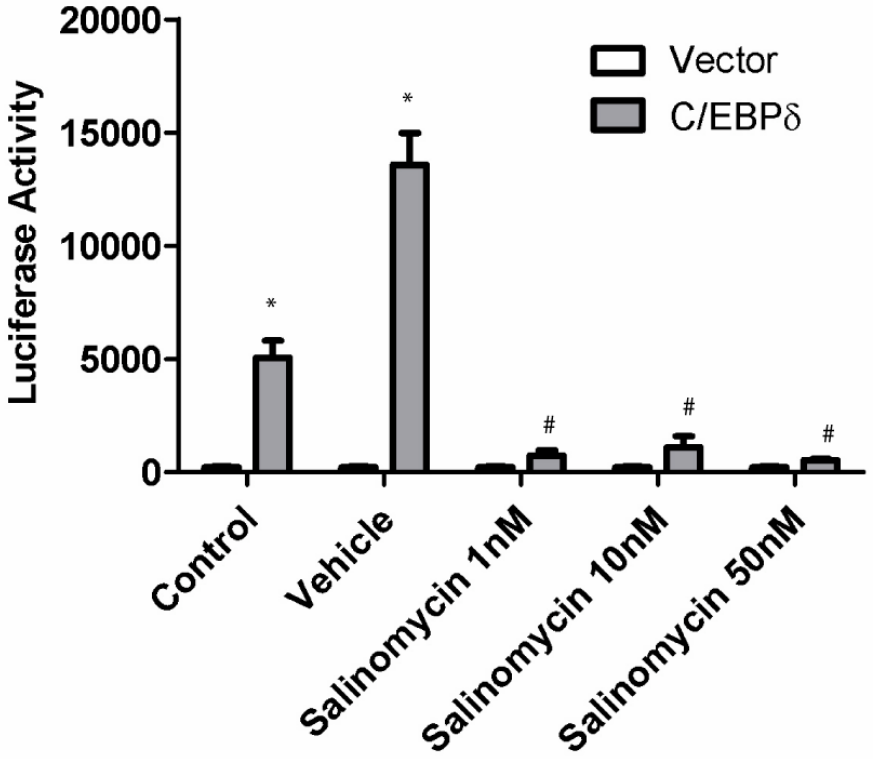

800
600
400
200
0
Luciferase Activity
Vector
C/EBPβ
*
*
#
#
#
Control
Vehicle
Salinomycin 1nM
Salinomycin 10nM
Salinomycin 50nM

Figure 5

e


20000
15000
10000
5000
0
Luciferase Activity
Vector
C/EBPδ
*
*
#
#
#
Control
Vehicle
Salinomycin 1nM
Salinomycin 10nM
Salinomycin 50nM

f

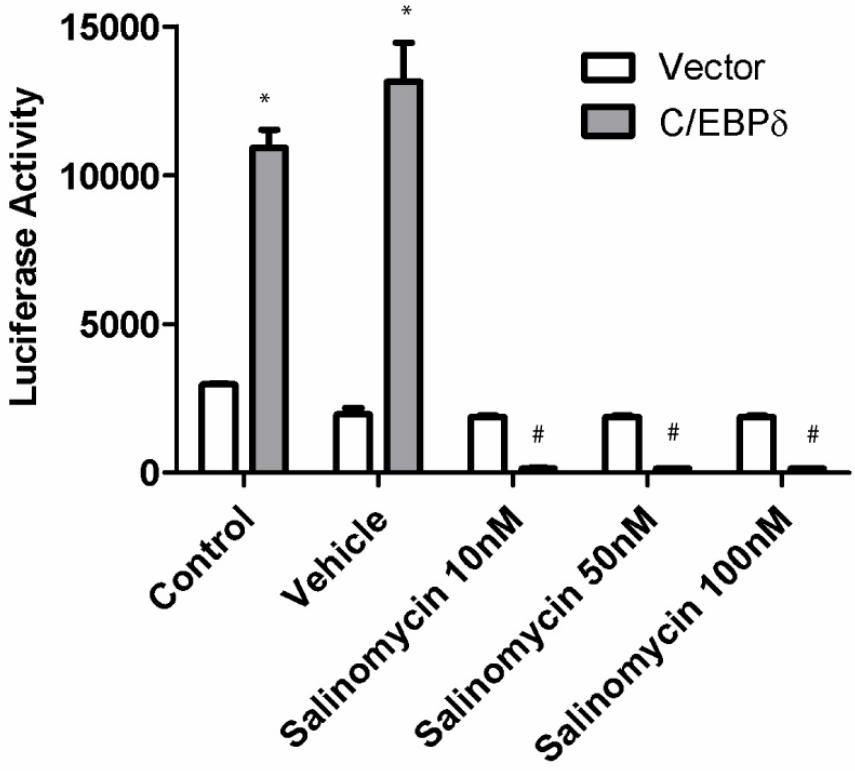
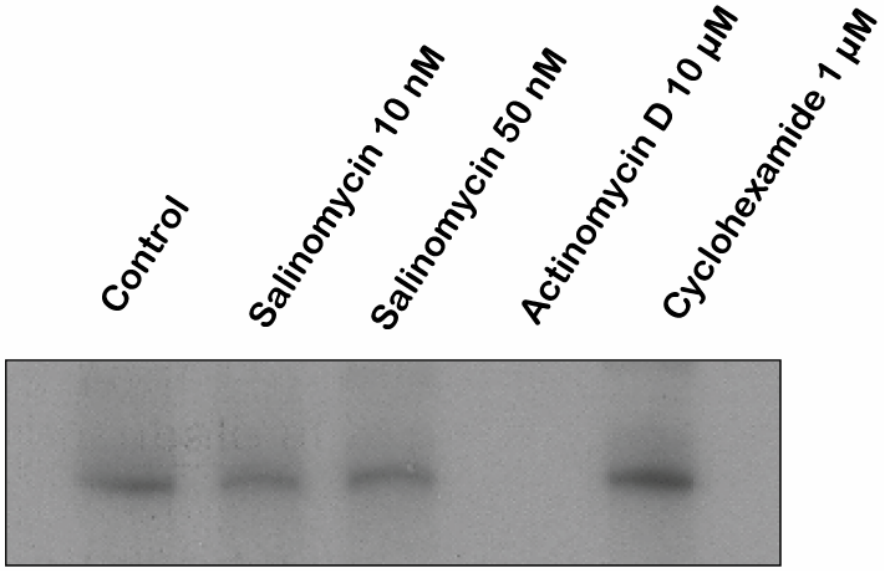

15000
10000
5000
0
Luciferase Activity
Vector
C/EBPδ
*
*
#
#
#
Control
Vehicle
Salinomycin 10nM
Salinomycin 50nM
Salinomycin 100nM

Figure 6

a


Control
Salinomycin 10 nM
Salinomycin 50 nM
Actinomycin D 10 μM
Cyclohexamide 1 μM

b

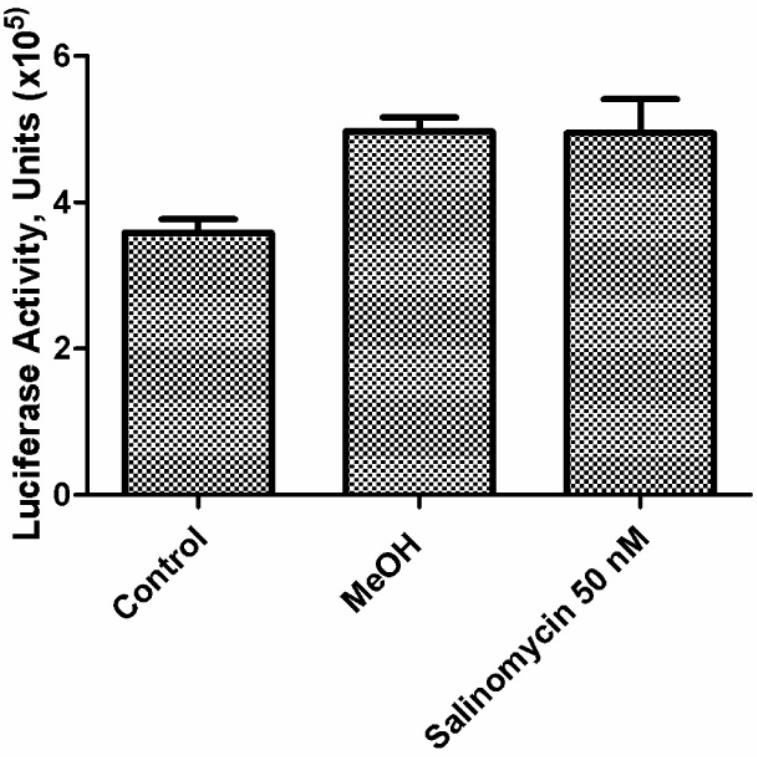

Luciferase Activity, Units (x10^5)
6
4
2
0
Control
MeOH
Salinomycin 50 nM

Figure 6

c

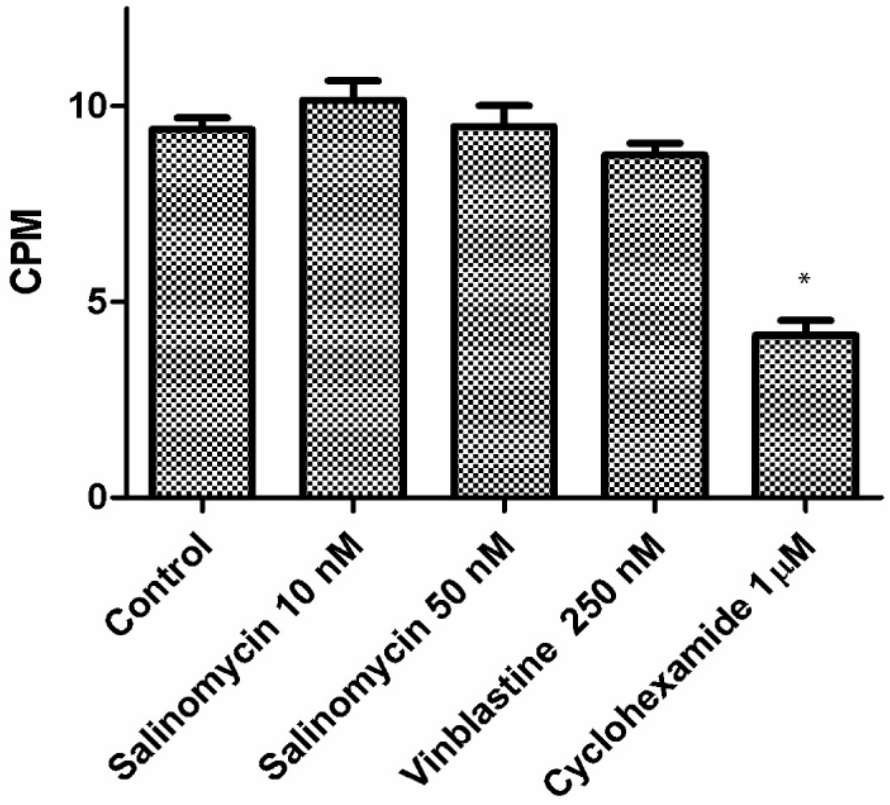
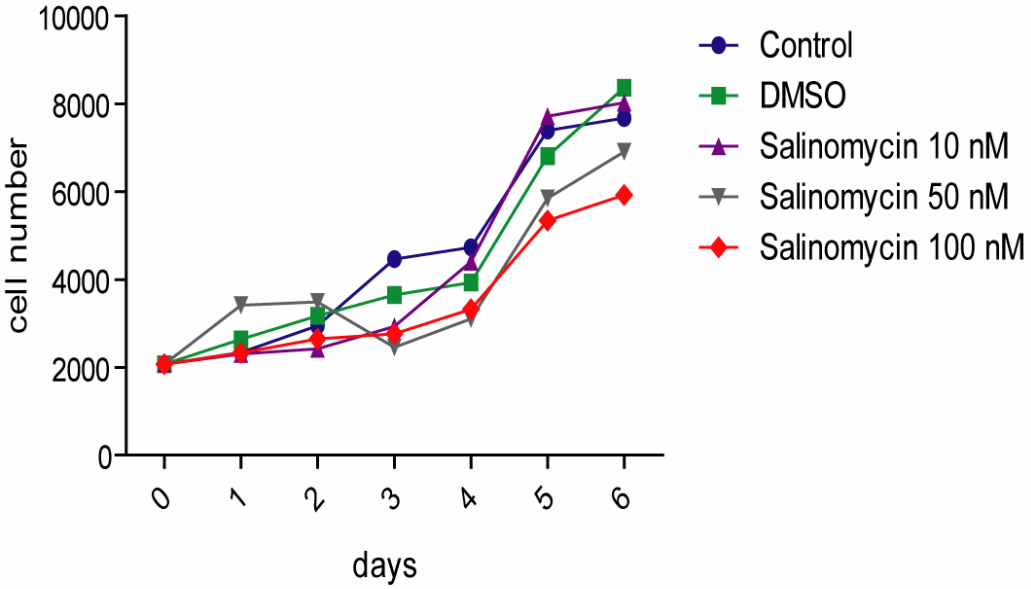

CPM
10
5
0
*
Control
Salinomycin 10 nM
Salinomycin 50 nM
Vinblastine 250 nM
Cyclohexamide 1 μM

Figure 7

a


10000
8000
6000
4000
2000
0
cell number
0
1
2
3
4
5
6
days
Control
DMSO
Salinomycin 10 nM
Salinomycin 50 nM
Salinomycin 100 nM

b

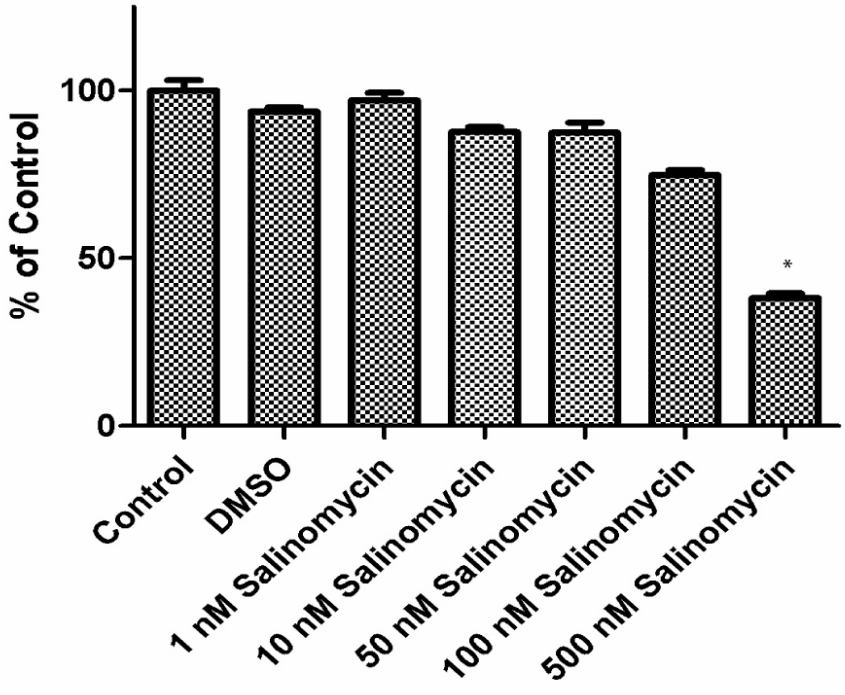
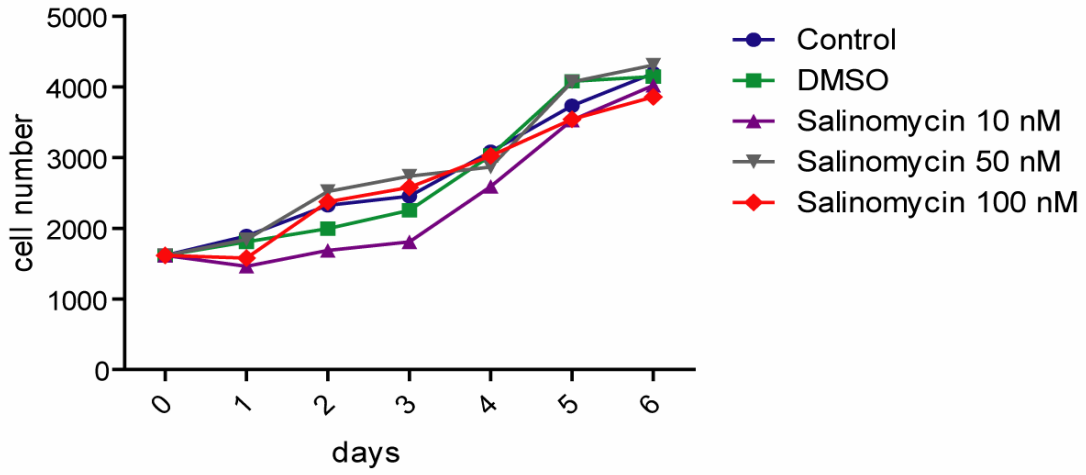
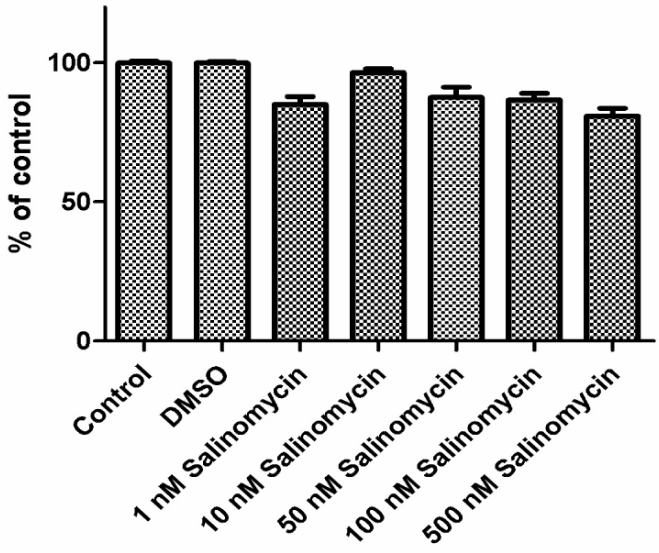

100
50
0
% of Control
*
Control
DMSO
1 nM Salinomycin
10 nM Salinomycin
50 nM Salinomycin
100 nM Salinomycin
500 nM Salinomycin

Figure 7

c


5000
4000
3000
2000
1000
0
cell number
0
1
2
3
4
5
6
days
Control
DMSO
Salinomycin 10 nM
Salinomycin 50 nM
Salinomycin 100 nM

d


100
50
0
% of control
Control
DMSO
1 nM Salinomycin
10 nM Salinomycin
50 nM Salinomycin
100 nM Salinomycin
500 nM Salinomycin

Chapter V. Conclusions

Below is a list of conclusions from the dissertation research:

Manuscript 1

1. Exposure of the chicken embryo to cyclic-AMP dependent protein kinase C subunit (PKAc) resulted in the inhibition of new blood vessel growth
2. Isozyme specific antibodies against the C subunit of PKA showed that Cα and Cβ were found in the conditioned media of HCT-116 and LnCAP cells
3. Extracellular PKA (ECPKA) in cultured cancer cells caused reduction of new blood vessel growth, comparable to the effects of purified PKAc
4. Both purified PKAc as well as ECPKA in the conditioned media of cultured cancer cells inhibited angiogenesis by the chicken embryo chorioallantoic membrane (CAM) assay

Manuscript 2

1. Gene expression profiling by DNA microarray demonstrated early induction of a transcription factor, zinc finger protein 68 (Zfp68), by treatment with prieurianin
2. Zfp68 inhibits transcriptional activity of C/EBPα, C/EBPβ and PPARγ, key regulators of adipocyte differentiation
3. Zfp68 is a nuclear localized protein and its overexpression seems to attenuate the OP9 cell potential to differentiate into mature adipocytes

Manuscript 3

1. Salinomycin inhibited the differentation of OP9 preadipocytes into mature adipocytes
2. Inhibition of differentiation by salinomycin in OP9 cells was not a result of drug induced apoptosis
3. Salinomycin attenuated rosiglitazone-induced OP9 adipocytes
4. Salinomycin inhibited C/EBPα-, β-, δ-, mediated transactivation from their respective response element driven reporters
5. Salinomycin did not inhibit transcription nor translation from a synthetic template

Chapter VI. Summary

This dissertation consists of three manuscripts: 1. Inhibition of Angiogenesis by Extracellular Protein Kinase A. 2. Molecular Regulation of Adipogenesis by Prieurianin. 3. Inhibition of Adipogenesis by Salinomycin

The major focus of the first part of this dissertation was to investigate the physiological role of extracellular protein kinase A (ECPKA) in angiogenesis and characterize the PKAc isozymes released by cancer cells. (Chapter 2, Manuscript 1). The presence of PKA activity in the extracellular surface of cells is an intriguing phenomenon that is not very well understood (Cho, Park, et al., 2000; Cvijic, et al., 2000; Kita, et al., 2004; H. Wang, et al., 2007). Moreover the discovery of ECPKA in the serum of cancer patients raised the question about its function in the tumorigenic development of cancer. It is known, that the successful growth of tumors is dependent on the process of vascularization (angiogenesis), therefore in this study, we attempted to further characterize the biological actions of extracellular protein kinase A as it relates to the vasculature. For this purpose, we applied the chicken embryo chorioallantoic membrane (CAM) assay to determine the role of PKAc and ECPKA from the conditioned media of cultured colon (HCT-116) and prostate (LnCAP) cancer cells, on new blood vessels formation. We hypothesized that the abnormal and ubiquitous secretion of ECPKA by cancer cells into the serum of cancer patients may

contribute to tumor progression and metastasis. However, our results showed unexpectedly that not only secreted ECPKA in the conditioned media of cultured cancer cells, but also purified PKAc inhibited angiogenesis by CAM assay. Exposure of the chicken embryo to various concentrations (10, 50, and 100 ng) of PKAc resulted in the inhibition of new blood vessel growth as evident by the lack of capillary growth. Furthermore, PKAc mediated inhibition of angiogenesis seemed to be comparable to that of thalidomide, a known anti-angiogenic compound. Therefore, it is counterintuitive that cancer cells release a constitutively active protein kinase that inhibits angiogenesis, which might actually impede their growth and metastatic potential, thus compromising tumorigenesis.

What is the physiological function of this secreted PKA in the plasma? While the role of the intracellular PKA holoenzyme in cell growth has been extensively investigated (Chin, et al., 2002), little is known, however, regarding the function of this recently identified secreted ECPKA. It was found that enhanced activity of the intracellular PKA promotes endothelial tube formation, thus facilitating angiogenesis (Hashimoto, et al., 2006). On the other hand it has also been shown that activation of intracellular PKA causes the phosphorylation of the transcriptional repressor Id1 (a major contributor to angiogenesis) (Volpert, et al., 2002), and disrupts its nucleocytoplasmic shuttling, thus inhibiting angiogenesis (Nishiyama, et al., 2007). It has been reported previously, that increase in extracellular cAMP inhibits angiogenesis in vitro (Furumichi, et al., 1992). Cyclic AMP is an intracellular second messenger, which regulates cell permeability, morphology, and growth. However, the molecular mechanism for the antiangiogenic action of cAMP was not determined. Perhaps an increase in intracellular cAMP may results in the activation of cAMP-

dependent protein kinase. These results partially correlate with our observation, that indeed PKA demonstrates anti-angiogenic potential.

Though others and we have shown that in normal individuals, levels of ECPKA activity in the peripheral blood are negligible (Cho, Park, et al., 2000; Cvijic, et al., 2000; Kita, et al., 2004; H. Wang, et al., 2007), we have not explored whether ECPKA might be secreted when a system is perturbed in normal subjects. Therefore, it is conceivable that intracellular PKA has an angiogenic function during development that is distinct and independent of the action of ECPKA. During normal embryonic development and through adult tissue repair processes, unwanted cells are eliminated by apoptosis. It has been shown that direct activation of PKA with cAMP or by overexpression of the PKA catalytic subunit induces apoptosis in the proliferation of endothelial cells hence inhibiting angiogenesis (Kim, et al., 2002). While we did not measure endothelial cell death in this study, it is possible that ECPKA inhibits angiogenesis by inducing endothelial apoptosis. Moreover, overexpression of the Cα subunit gene of PKA has been shown to increase the production of ECPKA (Cho, Park, et al., 2000), which might account for the inhibition of angiogenesis noted in our study, and is consistent in part with the observed endothelial cell death.

We currently do not understand the underlying reasons for the release of the free C subunit of PKA in cancer patients. Once secreted, does ECPKA play the role of a driver gene in cancer development, or is it merely a passenger gene (Greenman, et al., 2007)? For one, we speculate that the aberrant secretion of ECPKA might be an unintended consequence of tumor progression. Resultantly, the release of ECPKA into the serum of cancer patients elicits an autoimmune response, because PKA is normally localized inside the cell and the extracellular C subunit might be antigenic.

This hypothesis is supported by the discovery of autoantibody against ECPKA in the serum of cancer patients (Nesterova, et al., 2006). We surmise that the production of autoantibody against ECPKA rapidly neutralizes its activity, hence preventing it from inhibiting angiogenesis and acting on its extracellular substrates or targets.

Taken together, our results from chapter one, indicate that the C subunit of PKA is secreted by cancer into the extracellular milieu and demonstrates an anti-angiogenic property. Further investigation will elucidate the mechanism of the angiogenic events at the molecular level, however, these results suggest that extracellular PKA regulation may be applicable to the suppression of angiogenesis in tumorigenesis and may have significant clinical implications as a target of therapeutics in cancer. The ubiquitous nature of the release of ECPKA and its potential application as biomarker in cancer, and whether the aberrant release of ECPKA is just a bystander effect or that it might have important function in cancer development warrant further investigation.

The next part of this dissertation (Chapter 3, Manuscript 2) focused on investigating the significance of zfp68 expression in adipocyte differentiation by treatment with prieurianin. Prieurianin, a limonoid, extracted from citrus plants, has been shown to be an insect feeding deterrent (Koul, Daniewski, Multani, Gumulka, & Singh, 2003; Sarker, et al., 1997). These observations prompted us to ask whether the anti-feedant property of prieurianin can be exploited for the treatment of obesity. Zfp68 was detected in a microarray experiment originally designed to identify novel prieurianin target genes in preadipocyte differentiation. Zfp68 was found as an immediate-early gene to be markedly induced by prieurianin. Consistent with the microarray data, we observed modest induction of zfp68 by prieurianin in a dose and time-dependent manner once verified by polymerase chain reaction with reverse

transcription (RT-PCR) from OP9 stromal cells treated with prieurianin in a time course.

Zfp68 is a protein that contains 606 amino acids with 9 C_2H_2 zinc finger structures, and belongs to the superfamily of the C_2H_2 zinc finger protein family. C_2H_2 zinc finger domains were initially identified as DNA-binding domains and function as transcriptional regulators (Brown, 2005). The C_2H_2 zinc finger proteins are among the most abundantly represented proteins in the eukaryotic genome and the majority of the members belonging to this family are capable of binding to DNA in a sequence selective manner (Iuchi, 2001). The Krüppel-like protein superfamily is further separated into subfamilies depending on the presence of additional conserved domains. About one third of the Krüppel-like fingers contain the KRAB domain, which is characterized by the 75 amino acid conserved sequence present at the N-terminus of the zinc fingers (Bellefroid, Poncelet, Lecocq, Revelant, & Martial, 1991). Zinc finger protein 68 also contains KRAB domain divided into two subdomains: KRAB-A domain followed by the KRAB-B domain at the N-terminus and nine repeated zinc finger domains located in the C-terminus (Agata, et al., 1999).

It has been shown previously that zinc finger proteins have an enormous impact on regulation of adipocyte differentiation. Krox20 (EGR1) has been previously recognized to be necessary for adipogenesis (Chen, et al., 2005). Just recently Zfp423 was identified as a regulator of preadipocyte cell determination, representing a crucial determinant of preadipocyte commitment (R. K. Gupta, et al.). While little is known about Zfp68 it is noteworthy to investigate its function in adipogenesis.

It has been shown that Zfp68 may be a transcriptional repressor and the KRAB-A domain may be responsible for transcriptional repression (Agata, et al., 1999). Furthermore it was shown, that Zfp68 inhibits the transcription through its association

with the corepressor KAP-1 (KRAB-associated protein-1) (Agata, et al., 1999), which interacts with heterochromatin protein 1 (HP1) (Matsuda, et al., 2001). Since Zfp68 was induced by prieurianin, a potential anti-obesity drug, we reasoned that Zfp68 might be a master repressor of the transcriptional regulators of adipogenesis. Indeed, zfp68 inhibited the transcriptional activity of the adipogenesis master regulators including the CCAAT/enhancer binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor γ (PPARγ). Since it was demonstrated that only KRAB domain is responsible for repression, it may be useful in future studies to include progressive deletions of Zfp68, to show that indeed the KRAB domain is repression accountable.

In an attempt to investigate the anti-adipogenic effect of zfp68, we first wanted to validate its distribution. Numerous C_2H_2 type of zinc finger proteins have been demonstrated to localize in the nucleus and to act as transcription repressors (Skapek, et al., 2000). The transient transfection of the fusion protein Zfp68-GFP demonstrated it to be found in the nucleus, which is in agreement with previous reports (Matsuda, et al., 2001).

Since Zfp68 plays a critical role in the transcriptional repression of adipogenesis induced by prieurianin by inhibiting C/EBPα-, C/EBPβ-, and PPARγ-regulated gene expression during preadipocytes differentiation it is noteworthy to further elucidate the anti-adipogenic effect of zfp68 on adipocytes differentiation. As expected, zfp68-overexpressing cells showed a decrease in intracellular lipid accumulations as evidenced by reduced Nile Red or Oil Red O staining. Which lead us to the conclusion, that overexpression of zfp68 attenuated the OP9 cell potential to differentiate into mature adipocytes.

Transcription factors are necessary for adipogenesis. The regulation of gene expression during adipogenesis has been extensively studied. The adipogenic transcription factors C/EBPs (CCAAT/enhancer binding proteins) and PPARγ (peroxisome proliferator-activated receptor-gamma) play pivotal roles in the transcriptional cascade during adipogenesis process (Gregoire, 2001; Rajala & Scherer, 2003). CCAAT/enhancer binding protein-beta (C/EBPβ) is expressed immediately after the induction of differentiation, and then PPARγ and CCAAT/enhancer binding protein-alpha (C/EBPα) act synergistically to promote adipogenesis (Rosen, et al., 2002; Tang & Lane, 1999). If Zfp68 is an early transcriptional repressor of adipogenesis, it should inhibit the expression of the key adipogenic proteins. Hence, next we examined expression of molecular markers of terminal differentiation after transfection with Zfp68. Certainly, Zfp68 blocked differentiation by significantly suppressing the upregulation of endogenous C/EBPα and C/EBPβ, and PPARγ genes, correlating with results from the Nile Red and Oil Red O staining assay.

Adipogenesis involves a temporally regulated set of genes, therefore to investigate and characterize the regulation of Zfp68 expression by prieurianin it is crucial to understand the underlying transcriptional network. Over the past two decades, attention has centered on the role of the nuclear receptor PPARγ and the members of C/EBP family of transcription factors in adipogenesis (Rosen & MacDougald, 2006). However, the role of zinc finger protein 68 in adipocyte differentiation is not completely understood. In light of our data from chapter two, here we conclude that zfp68 is one of the immediate early genes that mediate the transcription inhibition of adipogenesis by prieurianin. Zfp68 inbibited the transcriptional activity of C/EBPα and C/EBPβ, and PPARγ genes in the reporter assay and the endogenous protein level

in adipocytes, meaning that Zfp68 can act as a negative regulator of a subset of genes involved in adipocyte differentiation and function. In addition, an overexpression of Zfp68 seems to attenuate adipogenic differentiation in OP9 cells. Although more studies are required to examine the effects and mechanisms of Zfp68 *in vitro* and *in vivo*, the elucidation of Zfp68 signaling events during adipogenesis might be an important area for future investigations.

In the last chapter of the dissertation (Chapter 4, Manuscript 3), we focused on characterizing the function of novel pharmacological agents on adipogenesis, namely ionophore antibiotics. Ionophore antibiotics are used in farming for the prevention of coccidiodomycosis in poultry and to alter gut flora in order to improve nutrient absorption in ruminants. They facilitate transport of ions across biological membranes by forming lipid-soluble complexes with mono or divalent cations. The non-therapeutic use of wide spectrum anitmicrobial agents including ionophores is a common practice in promoting growth and improving animal production. Since the use of ionophore antibiotics are used in such a large scale it raises the question about its potential effect on human health. Is it possible that the increase in production of antibiotics correlates with increased prevalence of obesity?

To answer this question we evaluated the role of three of the most commonly used antimicrobial agents (salinomycin, monensin and narasin) on adipogenesis using OP9 stromal cells. Monensin exhibits significant preference for Na^+, while salinomycin and narasin (4-methylsalinomycin) demonstrate favoritism toward K^+. With salinomycin significantly increasing body weight (62%) in chickens (Johansen, Bjerrum, & Pedersen, 2007), we rationalized that it might increase the number and/or the size of fully differentiated adipocytes. However, our work unexpectedly revealed

that both salinomycin and monensin inhibited the differentiation of OP9 cells, with salinomycin showing a greater potency over monensin. Concentrations of either 10 nM of salinomycin or 50 nM of monensin were able to block the differentiation of preadipocytes into mature adipocytes. These observations are in contrast to what was previously found, that increasing intracellular calcium (Ca^{2+}) by A23187 (calcium ionophore) in early stages of differentiation suppressed human adipocytes differentiation (Shi, Halvorsen, Ellis, Wilkison, & Zemel, 2000), similary to other observations in the murine adipocyte cell line (Draznin, et al., 1988). Although either Ca^{2+}, Na^{+} or K^{+} may be required for different actions, the ability of both monensin and salinomycin in inhibiting preadipocytes differentiation into adipocytes further suggest that the mechanism of inhibition of adipogenesis is probably independent of the presence of a specific alkali cation. These observations suggest further investigation of additional potential molecular targets that mediate the inhibition of adipogenesis by these antimicrobial agents.

It is noteworthy that under the culture conditions of the adipogenesis assay, inhibition of differentiation by salinomycin in OP9 cells was not a result of drug-induced apoptosis as indicated by the lack of annexin V binding to phosphatidylserine, compared to cells treated with doxorubicin. It has been shown that salinomycin is able to induce apoptosis in human cancer cells (Fuchs, et al., 2009), however the concentrations were up to 100-fold higher than those used in our study, which might explain the different observations.

Although both monensin (Na^{+}-) and salinomycin (K^{+}-specific) inhibited differentiation, salinomycin was the most potent inhibitor among the whole group of inopohores tested; therefore, we used it as the prototype for all consequent studies. Salinomycin is a 751 monocarboxylic polyether antibiotic isolated from *Streptomyces*

albus that reveals a unique tricyclic spiroketal ring system and an unsaturated six-membered ring in the molecule (Fuchs, et al., 2009). Salinomycin acts in diverse biological membranes including cytoplasmic and mitochondrial membranes as an ionophore with strict selectivity for alkali ions and a great preference for potassium (Mitani, Yamanishi, & Miyazaki, 1975; Mitani, et al., 1976). Therefore, salinomycin is known as an anticoccidal drug in poultry and fed to ruminants to improve nutrient absorption and feed efficiency (Callaway, et al., 2003).

In addition to these well known antimicrobial properties, recently salinomycin has been shown to be a selective and potent cytotoxic agent against mammary cancer stem cells (P. B. Gupta, et al., 2009). Therefore, we further investigated the cytotoxic effects of salinomycin in OP9 and L1 cells. Cell count and MTT assays showed that OP9 cells were more sensitive to the cytotoxic effects of salinomycin than L1 cells. We rationalized that the differential sensitivity to salinomycin is due to the presence of more stem or stem-like cells in the bone marrow derived OP9 mouse stromal cells, than in the mouse embryonic fibroblast 3T3-L1 cells (Green & Meuth, 1974; Wolins, et al., 2006). Moreover, OP9 cells have been demonstrated to differentiate more readily and with greater robust than 3T3-L1 cells. Therefore, the inherently larger subset of stem or stem-like cells within the population OP9 cells in culture, are selectively targeted by salinomycin, thus accounting for the observed differentiation sensitivity between OP9 and L1 cells.

Rosiglitazone, a member of the thiazolidinedione (TZD) class of anti-diabetic drugs, is an activator of PPARγ and known to induce adipogenesis (Sharma & Staels, 2007). TZDs can induce adipocyte differentiation in the absence of the standard adipogenic mixture in several preadipocyte cell lines (Tafuri, 1996). We evaluated the role of

salinomycin on rosiglitazone-induced adipocyte differentiation and found that salinomycin partially antagonized rosiglitazone-induced adipogenesis.

The transcription factors C/EBPα, β, and δ, and PPARγ are considered master regulators of adipogenesis (Lefterova & Lazar, 2009). Since salinomycin blocks rosiglitazone-induced adipocytes, we asked whether salinomycin might pharmacologically repress the transactivation potential of C/EBPs, using promoter reporter assay driven by the C/EBP response elements. Our results showed that salinomycin inhibited C/EBPα-, C/EBP β-, C/EBPδ–mediated transactivation from their respective response element driven reporters. In addition, salinomycin was able to fully decrease the transactivation potential from the PPARγ native gene promoter.

It was shown that mechanism of action of some other known antibiotics like: avilamycin, efrotomycin is to inhibit protein synthesis (Butaye, et al., 2003). Since the mechanism of action of salinomycin is not well studied, we evaluated its role on protein synthesis. However, salinomycin did not inhibit protein synthesis from a luciferase transcript neither in a wheat germ cell-free extract nor in ^{35}S-methionine incorporation assay. In view of the fact that salinomycin inhibited C/EBPs activated gene expression, we assumed that it could potentially be a transcription inhibitor. To determine whether salinomycin is a transcription inhibitor, we examined its effects on cell-free HeLa nuclear extracts driven *in vitro* transcription, and our results showed that salinomycin did not inhibit transcription from a synthetic DNA template.

The widespread use of ionophore antibiotics in animal husbandry and the concerns for such practice on human health prompted us to investigate the biological effects of these antimicrobials on adipogenesis. Our results from the last part of this dissertation revealed unexpectedly that these agents are potent inhibitors of preadipocytes differentiation into adipocytes, potentially via their repression of the transcriptional

activity of the master regulators of adipogenesis including C/EBPα, C/EBP β, and C/EBP δ. In summary, our results in last manuscript demonstrated the anti-adipogenic activity of some of the ionophore antibiotics on preadipocytes. These compounds, particularly salinomycin, also inhibit the transcriptional activity of the master regulators of adipogenesis. There is a tremendous need for some of the ionophore antibiotcs to be further developed as novel anti-obesity therapeutics and as pharmacological tools for probing the biology of adipose tissues.

Chapter VII. References

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