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## INHIBITION OF CHOLESTEROL SYNTHESIS BY POLICOSANOL

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ABSTRACT OF DISSERTATION

Subhashis Banerjee

The Graduate School  
University of Kentucky

2010

# INHIBITION OF CHOLESTEROL SYNTHESIS BY POLICOSANOL

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## ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Pharmacy  
at the University of Kentucky

By  
Subhashis Banerjee

Lexington, Kentucky

Director: Dr. Todd D Porter, Associate Professor of Pharmaceutical Sciences

Lexington, Kentucky

2010

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## ABSTRACT OF DISSERTATION

### INHIBITION OF CHOLESTEROL SYNTHESIS BY POLICOSANOL

Cholesterol is an essential component of the cell, but excessive blood levels are a major risk factor for the development of atherosclerotic plaques that can lead to heart disease and stroke, the foremost cause of premature death in Western societies. Policosanol, a mixture of very long chain alcohols derived from sugarcane, has gained considerable attention among the public as safe and effective means to reduce blood cholesterol levels, a belief based on some early clinical studies. My research investigates one possible mechanism by which policosanols might decrease blood cholesterol levels: the inhibition of cholesterol synthesis in the liver. Previous studies with cultured hepatoma cells have indicated that policosanols suppresses HMG-CoA reductase activity, the regulatory step in cholesterol synthesis, by activation of AMP-kinase, which then inactivates HMG-CoA reductase by phosphorylation. My studies have confirmed this activation of AMP-kinase both in hepatoma cells and in whole animals after intragastric administration of policosanols. The present studies were also undertaken to identify the upstream signaling mechanism by which policosanols activates AMP-kinase. Treatment of rat hepatoma cells with policosanols increased the amount of phosphorylated CaMKK, which can directly activate AMP-kinase, but had only a small effect on LKB1, the principal activator of AMP-kinase. Intragastric administration to mice similarly activated CaMKK, but not LKB1, in the liver. To determine if metabolism of policosanols was

necessary for activation of AMP-kinase, siRNA-mediated suppression of fatty aldehyde dehydrogenase, fatty acyl CoA synthase-4, or  $\beta$ -ketothiolase in hepatoma cells prevented the phosphorylation of AMP-kinase and HMG-CoA reductase by policosanol, indicating that metabolism of these very long chain alcohols to fatty acids and subsequent peroxisomal  $\beta$ -oxidation is necessary for the suppression of cholesterol synthesis. As the principal product of fatty acid  $\beta$ -oxidation is acetyl-CoA, further studies demonstrated that addition of acetate to cells similarly activated AMP-kinase and inactivated HMG-CoA reductase. This finding argues that the activation of AMP-kinase by policosanol results from the generation of excess acetyl-CoA via peroxisomal metabolism. Finally, although the intestine is a significant source of circulating cholesterol, policosanol was unable to activate AMP-kinase in the small intestine. These findings open new perspectives for the control of cholesterol synthesis by activators of AMP-kinase.

KEYWORDS: Cholesterol, AMP kinase, HMG-CoA reductase, Hepatoma cells, Policosanol.

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Student's Signature

May. 21, 2010  
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INHIBITION OF CHOLESTEROL SYNTHESIS BY POLICOSANOL

By  
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Dr. Todd D Porter  
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May. 21, 2010



DISSERTATION

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DISSERTATION

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2010

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Dedicated

To the Almighty who endowed me,  
To my parents and my sister who encouraged me,  
To my supervisor who enabled me.

## ACKNOWLEDGEMENTS

It gives me great pleasure to acknowledge all those people who have made my days as a research student so meaningful and enjoyable. The list is long and it is difficult to do justice to everyone, but this being part of the thesis that is read most often, I shall try to come up with as complete a list as possible.

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Last but not the least; I don't have words to express my love and gratitude for my parents, Dr. Debashis Banerjee and Dr. Sushma Banerjee. My parents left no stone unturned to provide me with the best and it is their sacrifice, blessings and hard work that have helped me to witness this day in my life. This is also an opportunity for me to thank my sister, Soumyaa Banerjee for all her love and encouragement.

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## CHAPTER ONE

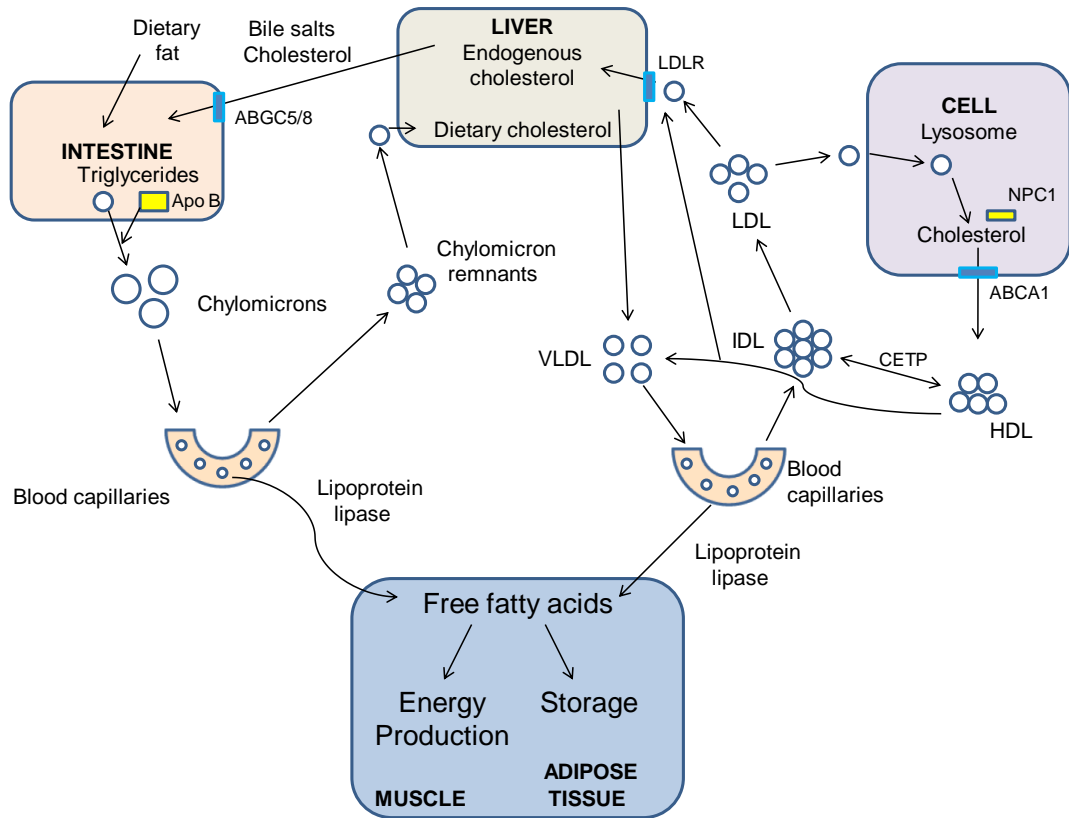
### BACKGROUND

Cholesterol has exerted a hypnotic fascination for scientists in the medical field and is the most highly decorated small molecule in biology (Brown and Goldstein, 1986). Cholesterol is a component of lipoprotein complexes in the blood and is thought to be one of the major causes of the formation of atherosclerotic plaques which lead to heart disease and stroke, the foremost cause of premature death in Western societies (Egan and Lackland, 1998; Franklin and, Sanders, 2000; American Heart Association, 2001). But, contrary to popular belief, cholesterol has a number of essential physiological functions. In many neurons, a myelin sheath, rich in cholesterol, provides insulation for more efficient conduction of impulses. Cholesterol is an important modulator of membrane fluidity in mammalian systems. It is a major constituent in the plasma membranes of animals and smaller quantities are found in the membranes of intracellular organelles. Plants contain no cholesterol but instead contain structurally related sterols, including stigmasterol and  $\beta$ -sitosterol. Within the cell membrane, cholesterol functions in intracellular transport, cell signaling and nerve conduction. Cholesterol is essential for the structure and function of invaginated caveolae and clathrin-coated pits (Rodal et al., 1999). Cholesterol-rich compartmentalized domains (rafts/caveolae) have been implicated in a variety of cellular processes, including signal transduction, endocytosis, transcytosis and cholesterol trafficking (Barnett-Norris et al., 2005).

Cholesterol is an amphipathic molecule. It consists of a polar hydroxyl head group at C-3 and a nonpolar steroid nucleus and hydrocarbon side chain at C-17. The biosynthesis of this 27-carbon compound, though highly complex and energy-expensive, is derived from the simple precursor, acetate. Elucidation of this pathway by Konrad Bloch and others in 1950s has led to our understanding of the transport of cholesterol, its receptor-mediated endocytosis, and the means by which intracellular cholesterol production is influenced by dietary intake.

Cholesterol is a precursor to bile acids (*e.g.*, cholate, glycocholate, taurocholate) and a variety of steroid hormones including testosterone (produced in testis), estradiol and progesterone (produced in ovaries and placenta), cortisol and aldosterone (produced in the cortex of the adrenal gland). Vitamin D<sub>3</sub> derived from 7-dehydrocholesterol is the precursor to calcitriol, which regulates calcium and phosphate metabolism.

Cholesterol and cholesteryl esters are carried in the blood as plasma lipoproteins. Very low-density lipoprotein (VLDL) carries cholesterol, cholesteryl esters, and triacylglycerols from the liver to other tissues, where the triacylglycerols are catabolized by lipoprotein lipase, converting VLDL to low density lipoprotein (LDL). The LDL is taken up by receptor-mediated endocytosis in the liver via the LDL receptor. A genetic deficiency of this receptor is associated with increased blood levels of LDL and risk of heart attack and stroke. High-density lipoprotein (HDL) serves to remove cholesterol from peripheral tissues, carrying it to the liver. A high level of HDL in the blood is associated with a decreased risk of cardiovascular disease; therefore, HDL has been dubbed the 'good cholesterol' as opposed to LDL, the 'bad cholesterol'. Cholesterol can be obtained from the diet and be synthesized in the liver. Dietary cholesterol is transported from the small intestine to the liver by chylomicrons. The homeostatic control mechanisms such as synthesizing new cholesterol, excreting cholesterol into the bile, and transforming it into bile acids operate to keep plasma cholesterol relatively constant, and all occur in the liver.



**Figure 1.1:** Cholesterol transport in the body

## Use of natural products to lower cholesterol

A reduced life span is an outcome associated with many prevalent diseases, including hypercholesterolemia, diabetes and obesity. In an effort to prevent these diseases, many researchers have looked into the identification of natural products and nutritional supplements that have cholesterol-lowering efficacies. There is evidence that soy protein, tea extracts, policosanol, guggulipids (Davidson and Geohas, 2003) and many other natural products can lower cholesterol levels (Chagan et al., 2002). Clinical trial evidence strongly supports the notion that both red yeast rice and black tea theaflavins may lower LDL (Becker et al., 2009; Davies et al., 2003). Also, there is strong clinical trial evidence suggesting that marine omega-3 fatty acids lower triglycerides (McGowan and Proulx, 2009).

Phytosterols, found in all vegetable foods and an essential part of Mediterranean diet, inhibit intestinal cholesterol absorption and have a serum cholesterol-lowering effect (Escuriol et al., 2009). They act by either a) attenuating the NPC1L1 gene expression which result in a lower cholesterol uptake from the lumen; b) lowering the cholesterol esterification rate by the ACAT2 (acyl-CoA cholesterol acyltransferase) and hence the amount of cholesterol secreted via the chylomicrons, and c) upregulating the expression of ABC-transporters ABCG5 and ABCG8 in intestinal cells, which result in an increased excretion of cholesterol by the enterocyte back into the lumen (Sanclemente et al., 2009).

Green tea catechins are also currently used as safe and effective lipid-lowering therapeutic agents, and reduce the risk of cardiovascular diseases (e.g., coronary heart disease) by lowering the plasma levels of cholesterol and triglyceride (Koo and Noh, 2007). Studies *in vitro* indicate that green tea catechins, particularly (-)-epigallocatechin gallate, interfere with the emulsification, digestion, and micellar solubilization of lipids, critical steps

involved in the intestinal absorption of dietary fat, cholesterol, and other lipids (Koo and Noh, 2007).

Soy protein isoflavones, in addition to lowering plasma cholesterol and triglycerides, downregulate hepatic SREBP-1 expression through an insulin-independent mechanism (Velasquez and Bhatena, 2007).

Garlic and garlic-derived compounds also lowers blood cholesterol levels through inhibition of cholesterol synthesis. Early studies on the inhibition of cholesterol synthesis by garlic indicated that inhibition of HMG-CoA reductase was the likely mechanism (Qureshi et al., 1983). However recently Singh and Porter, 2006 reported that diallyl disulfide, diallyl trisulfide, and allyl mercaptan present in garlic causes the accumulation of sterol 4 $\alpha$ -methyl oxidase substrates with radiolabelling studies coupled with gas chromatography–mass spectrometry indicating this enzyme as the principal site of inhibition in the cholesterologenic pathway by garlic. Their conclusion is also in accord with the view that 4-dimethylated sterols, including lanosterol and dimethylzymosterol, have been shown to promote the degradation of HMG-CoA reductase via an Insig-mediated pathway (Song et al., 2005). Garlic is an also effective inhibitor of squalene monooxygenase. Garlic compounds appear to bind to vicinal cysteines on squalene monooxygenase, and this inhibition can be prevented by thiols and reversed by dithiols (Gupta and Porter, 2001).

Resveratrol, a polyphenol found in grape skin and red wine, is beneficial in the prevention of some cholesterol-mediated diseases including cardiovascular disease (Leifert and Abeywardena, 2008). Pleiotropic effects include inhibition of oxidation of LDL, activating novel proteins that prevent cell senescence, e.g. Sirtuin 1 (Dohadwala and Vita, 2009), inhibition of specific cholesterol uptake/transporters, such as the Niemann-Pick C1-like 1 cholesterol transporter (Leifert and Abeywardena, 2008), and also inhibition of squalene monooxygenase in the squalene biosynthesis pathway (Laden and Porter, 2001).

Ginkgo biloba extract has been reported to decrease the total cholesterol content and inhibits the activity of HMG-CoA reductase and decreases cholesterol influx in cultured hepatocytes (Xie et al., 2009).

## **Policosanol lowers blood cholesterol**

Policosanol is a mixture of higher primary aliphatic alcohols obtained from sugar cane wax which have potent cholesterol lowering properties comparable to the low to medium dose statin therapy (Castaño et al., 2003). Policosanol is an attractive alternative for those patients who are in need of lipid lowering treatment but are reluctant to use, or unable to afford, chemically derived drugs. Lipid lowering effects of policosanol have been studied in a number of animal species including rodents and dogs. Serum cholesterol levels in pigs are significantly decreased by supplementation of the diet with policosanol (Cruz-Bustillo et al., 1991). At doses of 10-20 mg/day policosanol has been shown to lower total and LDL cholesterol by almost 30% and increases the level of good HDL cholesterol by almost 15%. The product is well tolerated and does not have any adverse effects (Gouni-Berthold and Berthold, 2002). In both short term (~12 weeks) and long term (up to 2 years) randomized placebo-controlled studies policosanol lowered LDL cholesterol in normocholesterolemic patients by an average of 33% (Gouni-Berthold and Berthold, 2002; Varady et al., 2003). Similar studies in people with hypercholesterolemia, hypertension, type 2 diabetes, elderly and post menopausal women resulted in LDL cholesterol reductions of 19-31% (Janikula, 2002). Policosanol did not significantly alter the triglyceride levels in any of the studies. In contrast to these studies in man, policosanol had no significant favorable effect in changing lipid levels in hamsters (Wang et al., 2003). Policosanol has shown a cholesterol lowering effect in rabbits and dogs (Arruzazabala et al, 1991, 1992, 1994), swine (Cruz-Bustillo et al, 1991) and monkeys (Rodriguez-Echenique et al, 1992). In monkeys there was also a significant reduction of spontaneous aortic atherosclerotic lesions in treated animals (Rodriguez-Echenique et al, 1994). However, several recent studies have been unable to demonstrate a significant reduction in serum cholesterol levels with policosanol treatment, calling into question the efficacy of this regimen (Kassis et al., 2009; McGowan and Proulx, 2009).



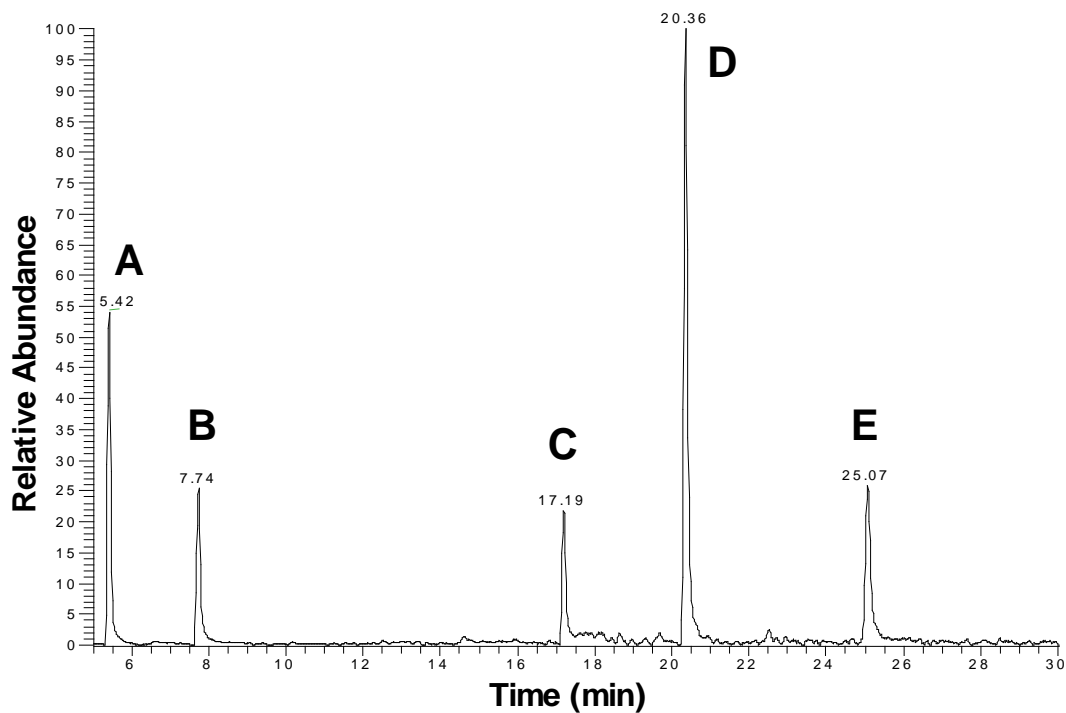
## **Policosanol decreases cholesterol synthesis**

A decrease in cholesterol synthesis is the principle mechanism by which policosanol lowers blood cholesterol as evidenced by several *in vitro* and *in vivo* studies. Policosanol administered orally inhibited hepatic cholesterol biosynthesis in rats with a 20% decrease in absolute rates of incorporation of <sup>3</sup>H-water into sterols (Menendez et al.,1996). LDL catabolism seems to be a primary mechanism by which policosanol lowers plasma cholesterol, as low density lipoprotein (LDL) processing is markedly enhanced initially. LDL binding, internalization and degradation were significantly increased after policosanol treatment (Menendez et al., 1994). Although a sterol-independent mechanism is quite possible for increased activity of LDL receptor, studies suggest that this is due to inhibition of cholesterol biosynthesis (Menendez et al, 1994). Cholesterol biosynthesis is decreased in a dose dependent manner from [<sup>14</sup>C] acetate and <sup>3</sup>H water but not from [<sup>14</sup>C] mevalonate when human fibroblasts was incubated with policosanol, suggesting a role for its modulation of HMG-CoA reductase activity. However, no evidence of competitive nor non-competitive inhibition of enzyme activity was observed in this study, thus negating it as a direct inhibitor of HMG-CoA reductase. When cells were grown in lipid-depleted media policosanol prevented the upregulation of HMG-CoA reductase activity (Menéndez et al., 2001a) but mRNA and protein levels were not measured in this study, making it difficult to determine if this decrease in reductase activity was due to increased degradation or decreased synthesis of the enzyme. Moreover, it remained possible that a metabolite of policosanol directly inhibits enzyme activity.

## Composition and metabolism of policosanol

Policosanol is a natural mixture of aliphatic primary alcohols isolated from sugarcane by hydrolytic cleavage and subsequent purification. It is designated as  $\text{CH}_3-(\text{CH}_2)_n-\text{CH}_2\text{OH}$ . Analysis of a commercially available 20-mg tablet of policosanol after extraction with methylene chloride and subjected to gas chromatography/mass spectrometry on an ion trap spectrometer showed the peaks for major components as illustrated in Fig 1.2 (Singh et al., 2006). The major components of policosanol are octacosanol ( $\text{C}_{28}$ , ~60%), triacontanol ( $\text{C}_{30}$ , 12-14%), and hexacosanol ( $\text{C}_{26}$ , 6-12%), while other alcohols (tetracosanol, heptacosanol, nonacosanol, dotriacontanol and tetratriacontanol) are minor components. Though the active components are unknown, researchers hypothesize that the inhibition of cholesterol synthesis is caused by the higher primary aliphatic alcohols and also by the presence of long chain fatty acids and chain-shortened metabolites. Studies with  $\text{C}_{24:0}$  and  $\text{C}_{26:0}$  acids have shown that these very long chain fatty acids are oxidized in peroxisomes, rather than mitochondria (Singh et al., 1987). They confirmed that acetate was the oxidation product from  $\text{C}_{24:0}$  whereas  $\text{C}_{26:0}$  and  $\text{C}_{26:0}$  CoA was converted to acetyl CoA, presumably obtained through  $\beta$ -oxidation. However, the study could not delineate clearly whether peroxisomes had a specific  $\beta$ -oxidation system to oxidize very long chain fatty acids or whether the peroxisomal  $\beta$ -oxidation long chain fatty acids system was solely responsible (Singh et al., 1987). A study on the biodistribution and metabolism of octacosanol in rats found it to be partly oxidized and degradation to fatty acids through  $\beta$ -oxidation (Kabir and Kimura, 1993). D-003, a mixture of very long chain saturated fatty acids isolated and purified from sugar cane wax, similarly increased serum HDL and significantly decreased serum total cholesterol and LDL in rabbits, and induced a dose-dependent inhibition in cholesterol biosynthesis (Menendez et al., 2004). The close similarity in chemical structures and a rapid and greater decrease in blood lipids with D-003 also suggested that  $\beta$ -oxidation of

policosanols to very long chain fatty acids may be necessary for their hypocholesterolemic actions (Menendez et al., 2004; Castano et al., 2005).



**Fig. 1.2:** Gas chromatogram of policosanols. Peaks A, B, C, D, and E were analyzed by mass spectrometry and shown to correspond to **A**, C<sub>16</sub> carboxylic (palmitic) acid; **B**, C<sub>18</sub> carboxylic (stearic) acid; **C**, C<sub>26</sub> alcohol (hexacosanol); **D**, C<sub>28</sub> alcohol (octacosanol); and **E**, C<sub>30</sub> alcohol (triacontanol). (Taken from Singh *et al.*, 2006.)

## **Regulation of HMG-CoA reductase activity**

HMG-CoA reductase (or 3-hydroxy-3-methyl-glutaryl-CoA reductase or HMGR) is an endoplasmic reticulum-bound enzyme that catalyzes the rate-limiting step in the mevalonate pathway which produces isoprenoids and, ultimately, cholesterol. HMG-CoA reductase contains eight transmembrane segments with the active site located in a long carboxyl-terminal domain in the cytosol (Roitelman et al., 1992; Istvan and Deisenhofer, 2000). It is an important enzyme in development and is the major drug target for cholesterol-lowering drugs. HMG-CoA reductase is reported to be present in all tissues, but highest expression occurs in the liver where it is subject to feedback regulation by cholesterol. HMG-CoA reductase is regulated at multiple levels *viz*, transcription, translation, degradation, and regulation of catalytic efficiency by phosphorylation. Regulation varies with species, with mice and hamsters exhibiting transcriptional control while rats show translational regulation (Ness and Chambers, 2000). Sterols affect both the transcriptional rate and translational efficiency, whereas hormones, most notably insulin and glucagon, affect both transcription and the phosphorylation state of the enzyme (Peffley and Gayen, 2003; Ness and Chambers, 2000).

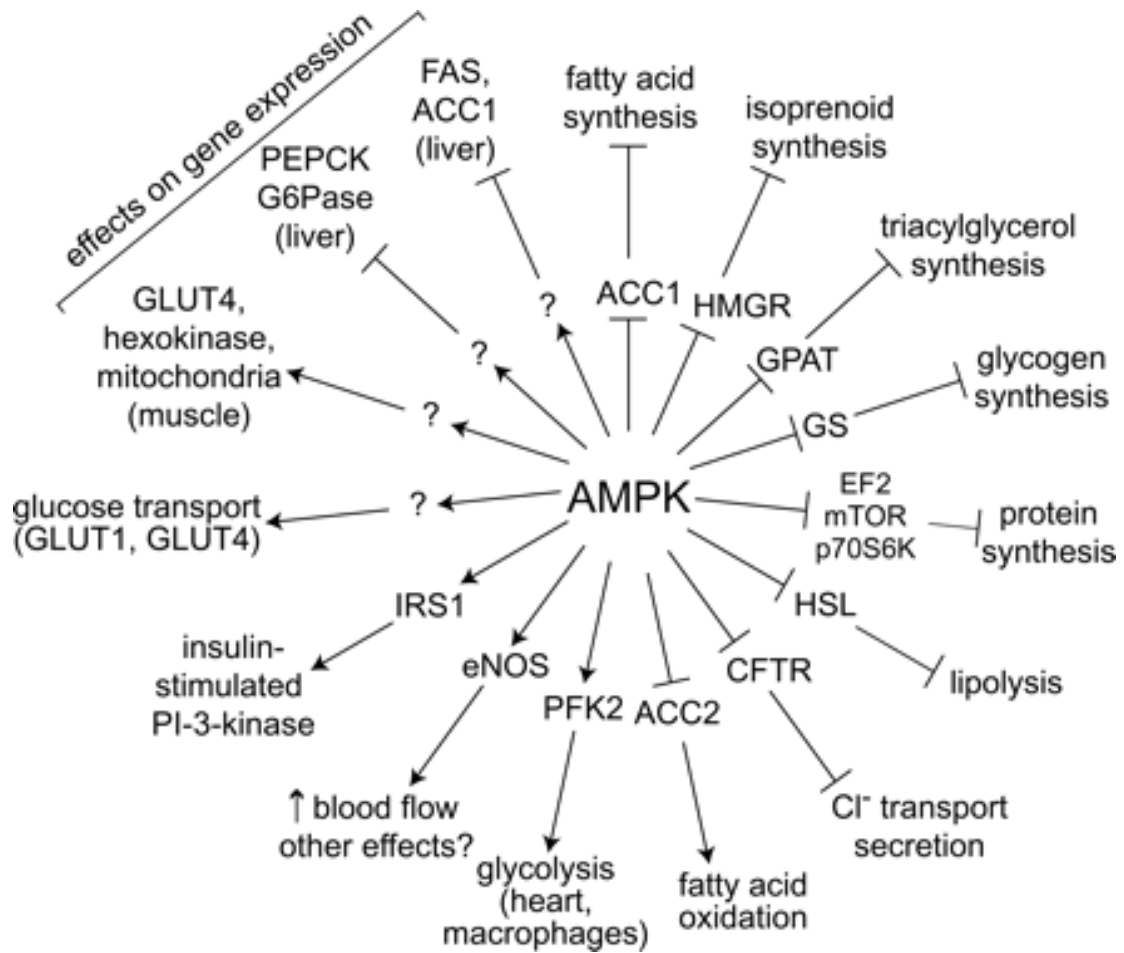
Transcriptional regulation of HMG-CoA reductase is both interesting and unusual, as illustrated by Dr. Michael S. Brown and Dr. Joseph L. Goldstein, who won the Nobel Prize in Physiology or Medicine in 1985 for their discoveries concerning "the regulation of cholesterol metabolism" (Goldstein and Brown, 1990). Transcription of the reductase gene is regulated by the sterol regulatory element binding protein (SREBP). This protein binds to the sterol regulatory element (SRE), located on the 5' end of the reductase gene. When SREBP is inactive, it is bound to two other proteins: SCAP (SREBP-cleavage-activating protein) and Insig1 in the ER or nuclear membrane. When cholesterol levels fall, Insig-1 dissociates from the SREBP-SCAP complex, allowing the complex to migrate to the Golgi apparatus, where SREBP is cleaved by S1P and S2P (site-1 and -2

protease), two enzymes that are activated by SCAP when cholesterol levels are low. The cleaved SREBP then migrates to the nucleus, where it binds to the SRE and transcription of genes like LDL receptor and HMG-CoA reductase is enhanced. If cholesterol levels rise, proteolytic cleavage of SREBP from the membrane ceases and any proteins in the nucleus are quickly degraded.

HMG-CoA reductase is also regulated through changes in the mRNA stability and translation. Translational efficiency of the reductase mRNA is significantly downregulated by dietary cholesterol as seen with decreased association of polysomes and subsequently decreased rate of initiation of protein synthesis (Chambers and Ness, 1997). Addition of oxysterols also leads to translational regulation of HMG-CoA reductase in Chinese Hamster Ovary cells (Leonard et al., 1994). The susceptibility of the reductase enzyme to the ER-associated degradation and proteolysis is increased with high levels of cholesterol. Helices 2-6 of the HMG-CoA reductase transmembrane domain sense the higher levels of cholesterol and lead to the surface exposure of lysine-248. The enzyme is then ubiquitinated by E3 ligase to undergo proteolytic degradation.

Microsomal reductase kinase, like HMG-CoA reductase, exists in active as well as inactive forms (Ingebritsen et al., 1978; Gibson and Ingebritsen, 1978) and can be regulated by phosphorylation (Beg et al., 1973). This kinase corresponds to AMP-kinase (Zammit and Easom, 1987). HMG-CoA reductase is primarily phosphorylated at Ser-872, close to the C-terminus and thus inactivated by the AMP-activated protein kinase, which senses AMP levels in the cell (Clarke and Hardie, 1990). AMP-kinase, unlike HMG-CoA reductase, is activated by phosphorylation by one or more upstream kinases at the threonine-172 position within the activation loop of the  $\alpha$ -subunit. AMP-kinase is activated by cellular stresses (eg. hypoxia, glucose deprivation, ischemia) and in response to a variety of hormones including glucagon, leptin and adiponectin (Hardie, 2003). AMP-kinase also inactivates acetyl-CoA carboxylase, the

regulatory step in fatty acid synthesis (Beg et al., 1987; Carling et al., 1987). The list of other targets for AMP-kinase or downstream processes that are affected is extensive, as shown in Fig 1.3 below.



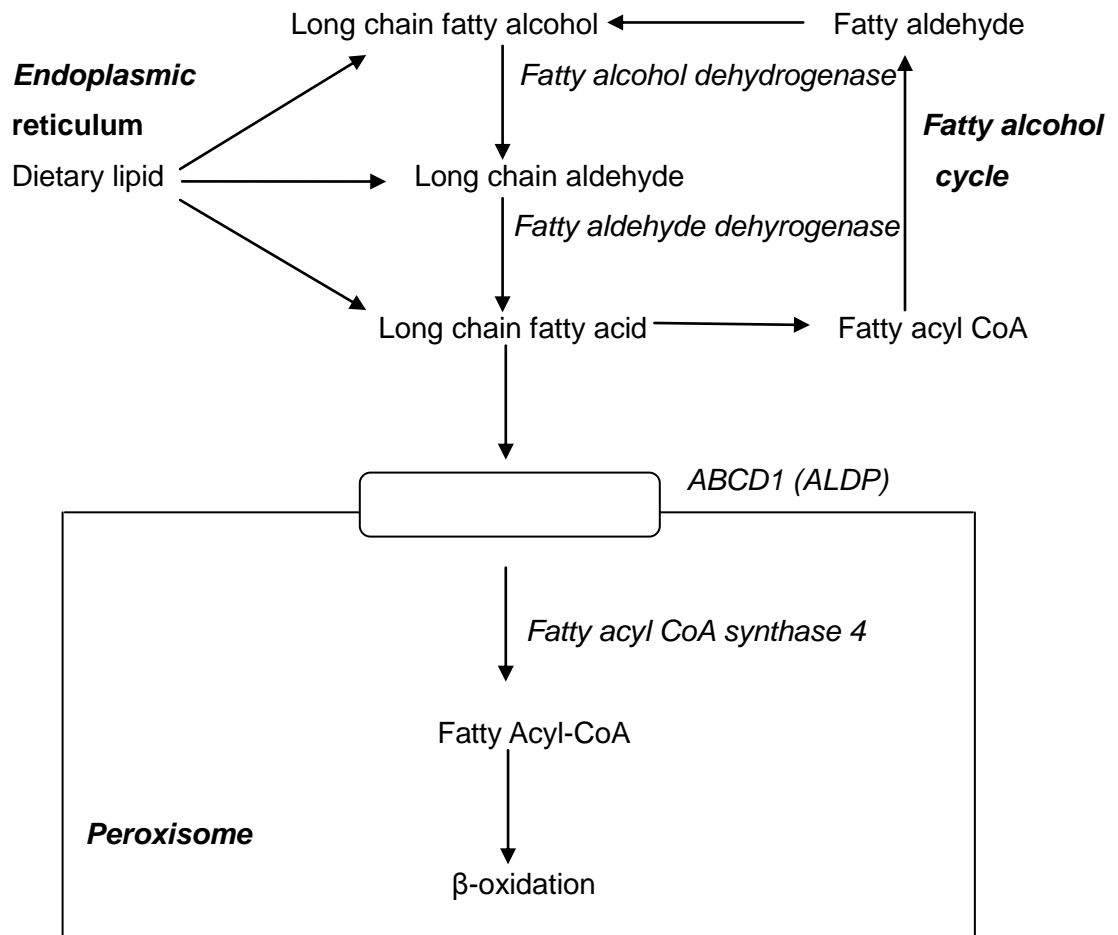
**Fig 1.3:** Known physiological target proteins and pathways regulated by the AMP-kinase system (modified and adapted from Hardie *et al.*, 2003).



Long chain fatty acids can stimulate the phosphorylation and activation of AMP-kinase (Clark et al, 2004; Carling et al., 1987) which may provide a possible route by which policosanol could activate AMP-kinase and repress cholesterol synthesis: policosanol may be oxidized in the cell to the corresponding fatty acid, which then directly, or through further metabolites, promotes the phosphorylation of AMP-kinase and subsequently, HMG-CoA reductase. A similar but more rapid onset of effects and a greater decrease in blood lipids were obtained with very long chain fatty acids (D-003) which also indicates that  $\beta$ -oxidation of policosanols to very long chain fatty acids may be necessary for their hypocholesterolemic actions (Menendez et al., 2004; Castano et al., 2005).

### **Very long chain fatty alcohol metabolism**

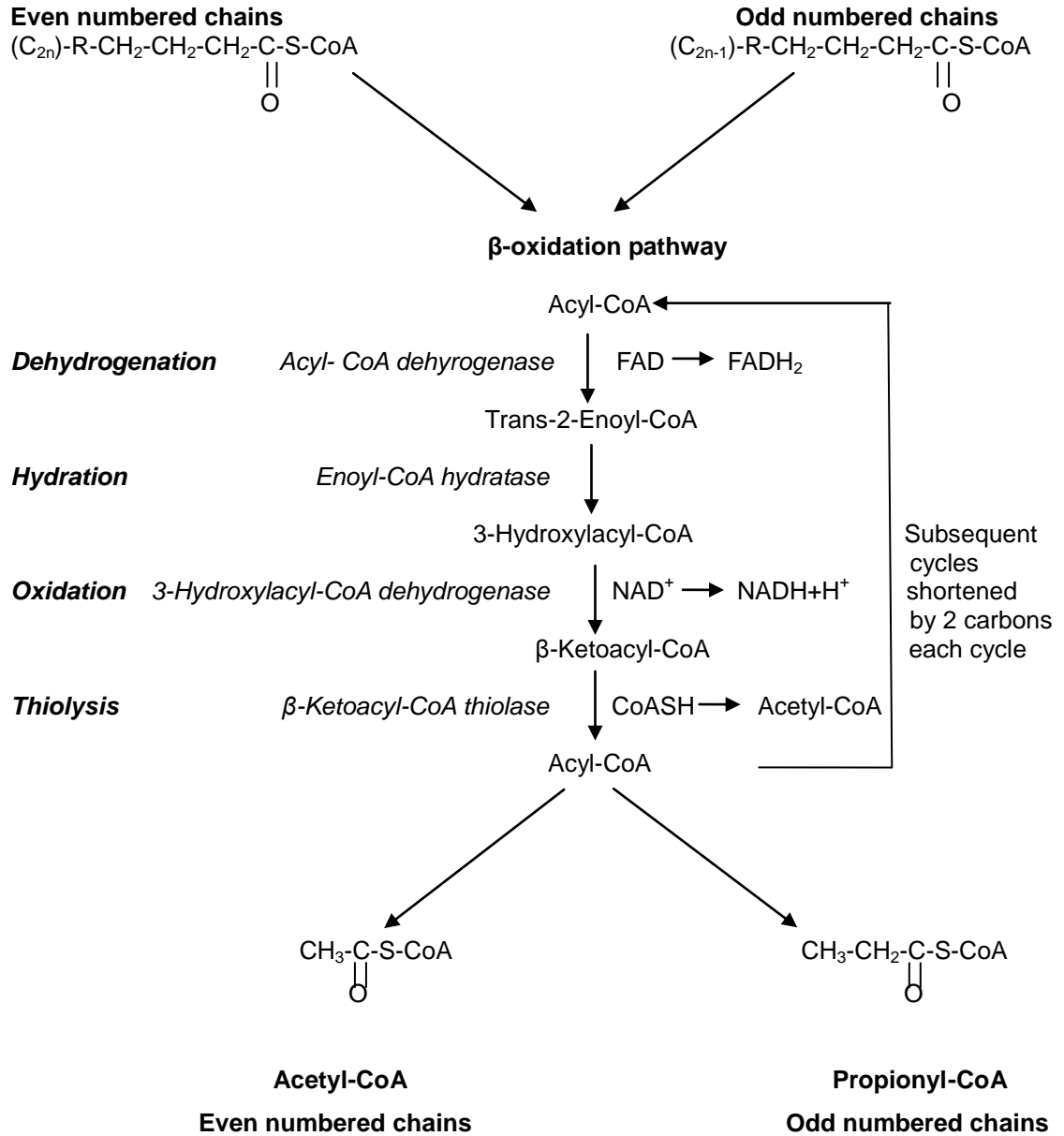
Very long chain fatty acids inhibit cholesterol synthesis in cell culture (Menendez et al., 2001a). Notably, peroxisomal diseases, including X-linked adrenal leukodystrophy and Sjögren-Larsson syndrome appear to result from defects in fatty alcohol and fatty acid metabolism (Hargrove et al., 2004; Rizzo et al., 2001; Rizzo and Craft, 2000). These diseases lead to a number of neurological and immunological disorders. Recent studies also indicate that very long chain fatty acids and cholesterol metabolism intersect at various points, particularly in regard to peroxisomal fatty acid oxidation and activation of PPAR $\alpha$  transcriptional system. Synthesis of ether-phospholipids, important in lipid turnover, and oxidation to very long chain fatty acids are the two major pathways for utilization of long-chain alcohols. Interconversion between very long chain fatty alcohols, aldehydes and acids have also been reported (Rizzo et al., 1987). However, the dominant route for metabolism of fatty alcohols is oxidation to fatty acids followed by  $\beta$ -oxidation, as is evidenced by the accumulation of long chain alcohols when fatty aldehyde oxidoreductase is deficient (Hargrove et al., 2004).



**Fig 1.4:** Fatty alcohol cycle and peroxisomal  $\beta$ -oxidation.

Mixtures of VLCFA from sugarcane inhibit cholesterol synthesis in fibroblasts (Menéndez et al., 2001b) and also inhibit lipoprotein oxidation *in vitro* (Menéndez et al., 2002). A reciprocal relationship between cholesterol accumulation and metabolism of VLCFA may exist as viewed from patients with defective peroxisomal activity. Cholesterol depletion also upregulates VLCFA metabolism in cultured human fibroblasts and monocytes from patients with X-linked adrenoleukodystrophy (Weinhofer et al., 2002).

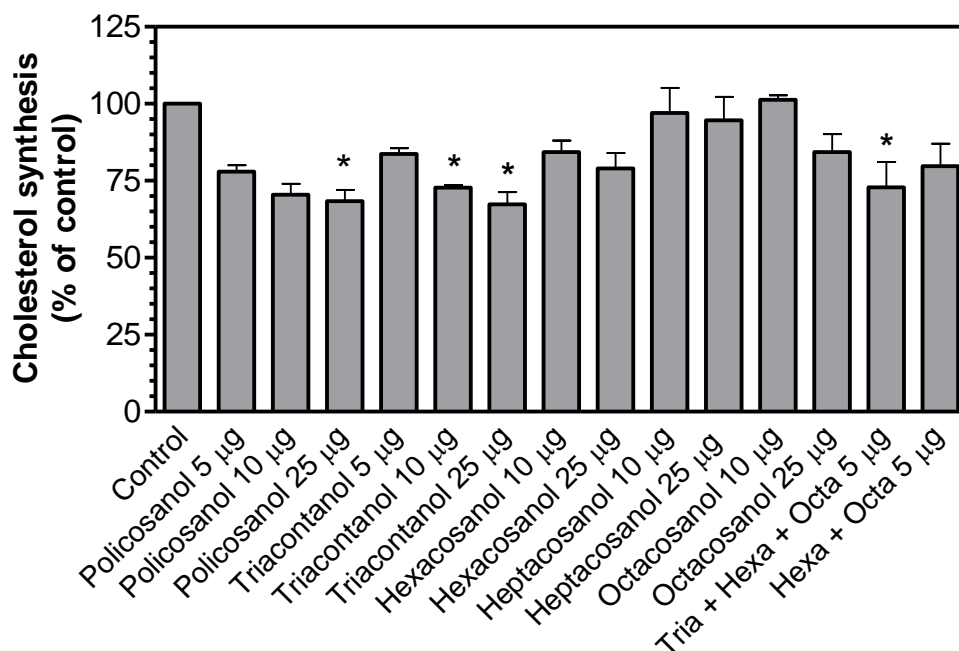
Animals, many protists, and some bacteria utilize oxidation of long chain fatty acids to acetyl-CoA as a central energy-yielding pathway, driving ATP synthesis. In vertebrate animals ketone bodies are formed in the liver whereas in plants the acetyl CoA is used as a biosynthetic precursor and secondarily as a fuel. Fatty acid oxidation occurs primarily in the mitochondria but peroxisomes of plants and animals also carry out  $\beta$ -oxidation. Fatty acid  $\beta$ -oxidation involves a repeating sequence of four reactions to remove each acetyl-CoA unit from the carboxyl end of saturated fatty acyl-CoAs: 1) dehydrogenation of the  $\alpha$ - and  $\beta$ -carbons by the FAD-linked acyl-CoA dehydrogenases; 2) hydration of the resulting trans- $\Delta^2$  double bond by enoyl-CoA hydratase followed by 3) the oxidation of the resulting L-  $\beta$ -hydroxyacyl-CoA by NAD-linked  $\beta$ -hydroxyacyl-CoA dehydrogenase, and finally 4) thiolysis by acyl-CoA acetyltransferase (thiolase) molecule to form acetyl-CoA and an acyl-CoA shortened by two carbon atoms. Fatty acids having an odd number of carbon atoms give rise to acetyl CoA (two carbon atoms) and propionyl CoA (three carbon atoms) in the final round of fatty acid degradation.



**Fig 1.5:** β-oxidation of fatty acids

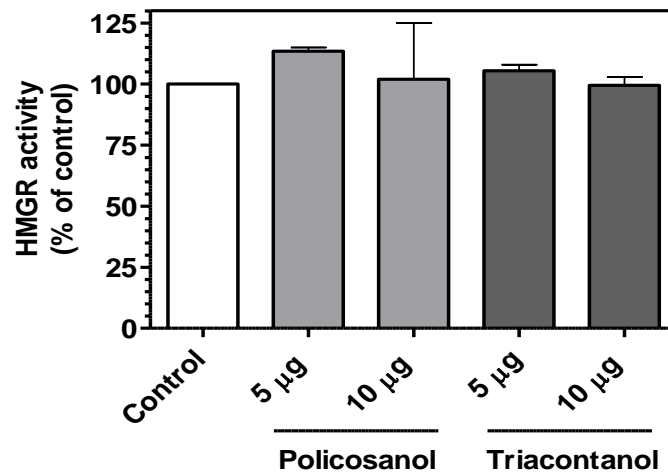
## Inhibition of cholesterol synthesis in hepatoma cells by policosanol

Previous work by Singh et al. (2006) confirmed that policosanol at physiologically relevant concentrations (<10 µg/ml) was able to inhibit cholesterol synthesis in hepatoma cells. They identified triacontanol, a C<sub>30</sub> alcohol, to be the most active component, although octacosanol is the principal component of policosanol. Policosanol was not toxic to cells even at doses up to 50 µg/ml as determined by trypan blue exclusion and lactate dehydrogenase assays.



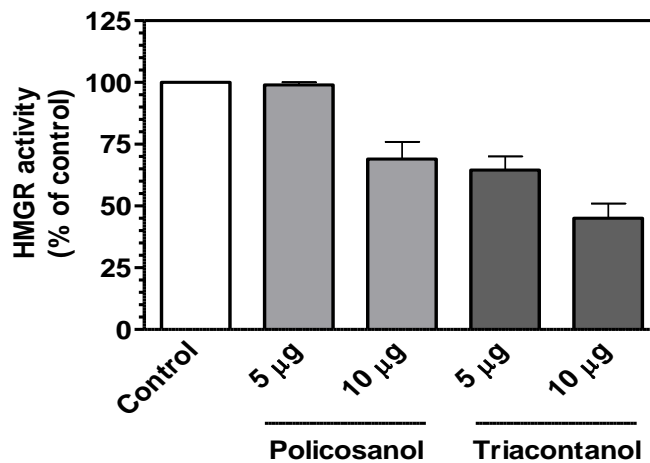
**Fig 1.6:** Inhibition of cholesterol synthesis by policosanol and its components. Cholesterol synthesis from <sup>14</sup>C-acetate was determined after 3 h in the presence of policosanol or its principal components. Values represent the mean and standard error of 2-5 experiments carried out in duplicate. Asterisk (\*) indicates statistical significance with respect to untreated cells as determined by one-way ANOVA with Tukey's post-hoc test. (Taken from Singh *et al.*, 2006.)

Policosanol decreased cholesterol synthesis from [ $^{14}\text{C}$ ] acetate but had no effect on cholesterol synthesis from [ $^{14}\text{C}$ ] mevalonate (Singh et al., 2006). This finding was reported earlier by Menendez et al. (2001a), indicating that policosanol acts at or above HMG-CoA reductase. Neither policosanol nor triacontanol inhibited HMG-CoA reductase when added to rat liver microsomes, indicating that there was no direct inhibition of HMG-CoA reductase by these alcohols.



**Fig 1.7:** Effect of policosanol and triacontanol on HMG-CoA reductase activity. HMG-CoA reductase activity was measured in microsomes prepared from rat liver to which policosanol or triacontanol was added as indicated. (Taken from Singh *et al.*, 2006.)

However, HMG-CoA reductase enzyme activity was decreased by almost 55% when hepatoma cells, rather than subcellular preparations, were incubated for 3 hrs with policosanol or triacontanol. These results suggest that either policosanol is metabolized to an active inhibitor or that it reduces the expression of HMG-CoA reductase by decreasing transcription and translation or by enhancing the degradation of the enzyme.



**Fig 1.8:** Effect of policosanol and triacontanol on HMG-CoA reductase activity. HMG-CoA reductase activity was measured in lysates prepared from hepatoma cells and incubated with the indicated concentrations of policosanol or triacontanol. (Taken from Singh *et al.*, 2006.)

Policosanol and triacontanol did not decrease HMG-CoA reductase protein levels over the 3 hr period of the experiment, though there was a marked decrease in activity of the enzyme. As HMG-CoA reductase protein levels were not changed by policosanol treatment, the possibility lies that policosanol inactivates HMG-CoA reductase by promoting its phosphorylation by one of the three protein kinases located upstream in the signaling pathway (Beg et al., 1987).



## CHAPTER TWO: EXPERIMENTAL RATIONALE

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the third enzyme and the regulatory step in the cholesterol biosynthesis pathway. Although the most common means to reduce blood cholesterol levels is to inhibit the activity of this enzyme directly, it may also be possible to suppress the activity of this enzyme indirectly by promoting its phosphorylation. Indeed, policosanol appears to decrease HMG-CoA reductase activity by activating AMP-kinase (Singh *et al.*, 2006), the principal regulatory kinase for HMG-CoA reductase (Clarke and Hardie, 1990). The mechanism by which policosanol activates AMP-kinase is the subject of this research, and one question addressed is whether policosanol requires metabolism to generate an active metabolite. Notably, D-003, a mixture of very long chain fatty acids, also suppresses HMG-CoA reductase activity (Menendez *et al.*, 2001b), raising the possibility that the long chain primary alcohols of policosanol undergo oxidation to the corresponding fatty acid, and perhaps subsequent peroxisomal  $\beta$ -oxidation, to form the active metabolite which activates the AMP-kinase pathway. Hence, the purpose of this study is to confirm that policosanol activates AMP-kinase and leads to the phosphorylation and inactivation of HMG-CoA reductase, to define the upstream signaling pathway or pathways through which policosanol or its active metabolite(s) activates this central regulatory kinase, and to determine if policosanol, administered orally by gavage, activates these pathways *in vivo*.

## Specific Aims

**Specific Aim 1:** Determine the mechanism by which policosanol promotes the phosphorylation (and suppression) of HMG-CoA reductase. This aim has two parts: 1) To identify the upstream kinase(s) that are activated by policosanol and lead to AMP-kinase phosphorylation and activation; and 2) to determine if policosanol must undergo metabolism via the fatty alcohol cycle and peroxisomal  $\beta$ -oxidation to activate AMP-kinase. To determine if upstream signaling pathways are involved, the phosphorylation states of different kinases known to be upstream of AMP-kinase (CaMKK, LKB1 and PKC $\zeta$ ) will be determined. The possibility that policosanol requires metabolism, including peroxisomal  $\beta$ -oxidation, will be tested through the use of RNAi to suppress the expression of enzymes in the fatty alcohol-peroxisomal  $\beta$ -oxidation pathways. If metabolism is required these experiments should allow the identification of the metabolite that leads to the activation of AMP-kinase.

**Specific Aim 2:** Determine if policosanol, administered to mice orally by gavage, results in the activation of AMP-kinase and the phosphorylation of HMG-CoA reductase in the livers of these animals. These studies will determine the dose necessary for activation and determine if the upstream kinases are the same as those identified in the hepatoma cell culture studies of Aim 1. Lastly, because the intestine is a significant source of circulating cholesterol, the effect of orally administered policosanol on intestinal AMP-kinase and HMG-CoA reductase phosphorylation will be determined. These *in vivo* studies will extend and confirm the corresponding *in vitro* studies described in Specific Aim 1 and, if successful, will provide support for the use of policosanol for the treatment of hypercholesterolemia in man.

## CHAPTER THREE: MATERIALS AND METHODS

### Chemicals

The commercial policosanol preparation Lesstano<sup>60</sup> was obtained from Garuda International, Inc. (Lemon Cove, CA). Dulbecco's modified media (DMEM), penicillin-streptomycin-glutamine (PSG, 100X), fetal bovine serum (FBS), lipoprotein-depleted serum, and trypsin were purchased from Invitrogen (GIBCO, Carlsbad, CA). Acadesine (AICAR), 1,1-dimethylbiguanide hydrochloride (metformin), ionomycin, sodium acetate, and protease inhibitor cocktail were obtained from Sigma. HALT phosphatase inhibitor and the BCA protein assay kit were purchased from Roche Diagnostics and Pierce Biotechnology (Rockford, IL, USA) respectively. McA-RH7777 rat hepatoma cells were obtained from American Type Culture Collection (Manassas, VA) and used between passages 12 and 22.

### Policosanol

**Chemical components of policosanol:** LesstanoL® brand policosanol contains naturally-occurring waxy alcohols (fatty alcohols) derived from sugarcane wax. Garuda's LesstanoL® Policosanol 60 (OCTA-60) contains a minimum of 97% policosanol of which 65.4% is 1-octacosanol along with other higher aliphatic alcohols including 1-hexacosanol (5.6%), 1-triacontanol (16.5%), 1-tetracosanol (2.2%) and 1-dotriacontanol (5.1%).

**Preparation of policosanol:** 20 mg of policosanol was dissolved in 10 ml of 50% ethanol with sonication for 30 min with intermittent vortexing. The solution was aliquoted and stored at -20°C, and added directly to media or buffer upon use.

## Cell culture

McA-RH7777 rat hepatoma cells between passages 12 to 22 were cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum and 1x Penicillin–Streptomycin (Invitrogen) in six-well plates at 37°C under a humidified atmosphere of 5% CO<sub>2</sub>. After 48 h, the medium was replaced with fresh medium with the addition of policosanol (10-25 µg/ml in 50% ethanol) and incubation was continued for 3 h. Control cells received an equal volume of 50% ethanol.

**Preparation of lysates:** Cells were washed once with phosphate-buffered saline (pH 7.4) scraped from the plates, pelleted by low-speed centrifugation and lysed by two cycles of freeze-thawing in 0.25 M Tris buffer (pH 7.5) containing protease and phosphatase inhibitors at 2x standard concentration (Roche Diagnostics). The lysates were cleared by centrifugation (18,300xg, 10 min, at 4°C) and the supernatant was stored in aliquots at -80°C.

## Animals

Experiments involving the use of animals were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Kentucky and were in accordance with all policies for the use and care of laboratory research animals as stipulated by the NIH. Female C57BL/6 mice (~15-17 grams) were used in the studies. Seven-to-eight week-old animals were obtained from Jackson laboratories (Bar Harbor, ME) and maintained in a temperature-, humidity-, and light- controlled facility (70-72°F, 48-52% humidity, 12-hr light/dark cycle) and were allowed free access to water and food. The animals were allowed to adjust for 1 week before beginning treatments. At 12 hours before the experiment, food was withdrawn and mice in groups of 5 were gavaged with 150 µl of policosanol in 50% ethanol in doses of 25 mg/kg, 50 mg/kg and 100 mg/kg body weight. Control animals received

an equal volume of 50% ethanol only. At specific time points mice were euthanized by CO<sub>2</sub> asphyxiation and the liver and intestine promptly removed for processing; a portion of each was snap-frozen in liquid nitrogen and stored at -80°C until used.

**Preparation of tissue homogenates:** Liver and intestine homogenates were prepared as follows: Approximately 25 mg of tissue was transferred into 4 volumes of chilled RIPA homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) and homogenized using a Potter-Elvehjem homogenizer. Protease and phosphatase inhibitors were used at 2× standard concentration (Roche Diagnostics). The samples were then subjected to two cycles of freeze–thawing and then cleared by centrifugation (18,300×g, 10 min, 4°C). The supernatant was stored in aliquots at -80°C. Protein concentration was determined by BCA assay (Pierce Biotechnology).

### **Gel electrophoresis and immunoblotting**

Thirty micrograms of protein as a cell lysate or tissue homogenate was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8% gels and electroblotted to nitrocellulose. The membrane was blocked with 0.05% Tween-20 and 5% defatted milk for 1 h at room temperature and then incubated in this same buffer with rabbit antibody to total AMP kinase (anti-AMPK α-pan, 1:2000; Upstate USA) or to phosphorylated AMP-kinase (anti-phospho-AMPKα, 1:500; Upstate USA) overnight at 4°C with gentle shaking. The immunoblot was developed with a secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature and the chemiluminescent image (Supersignal West Pico Chemiluminescent Substrate, Thermo Scientific) captured by autoradiography on a Kodak Image Station or on film. Band intensity on film was measured with Image J software on the

scanned image with background subtraction. The same procedure was followed for detecting phosphorylated HMG-CoA reductase (anti-phospho-HMG-CoA reductase, 1:500; Millipore USA) and phosphorylated PKC $\zeta/\lambda$  (anti-phospho-PKC $\zeta/\lambda$ , 1:500; Cell Signaling USA).

### **Immunoprecipitation**

To determine the level of phosphorylation of the kinases LKB1 and CaMKK, cell lysates or tissue homogenates were incubated for 1 h at 4°C with 20  $\mu$ l of mouse monoclonal antibody to phosphoserine/phosphothreonine/phosphotyrosine (Abcam). Antibody conjugates were precipitated with 35  $\mu$ l of protein A or G Plus-Agarose from Calbiochem (EMD Chemicals) for 1 h at 4°C by following an Amersham Biosciences immunoprecipitation protocol, as follows. After centrifugation at 12,000 $\times$ g for 20 sec at 4°C, the immunoprecipitated phosphoproteins were released from the agarose beads by boiling in 25  $\mu$ l of 2X gel loading buffer [0.5M Tris-HCl (pH 6.8), 4.4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, and bromophenol blue in distilled/deionized water] at 95°C for 4 min. After removal of beads by centrifugation at 12,000 $\times$ g for 20 sec at 4°C, the phosphoproteins were fractionated by SDS-gel electrophoresis and electroblotted to nitrocellulose as described above. Phosphorylated protein content was detected and quantified with an antibody to the total LKB1 (anti-LKB1, 1:1000; Sigma USA) and total CaMKK (anti-CaMKK, 1:1000; BD Transduction USA) followed by a secondary antibody conjugated with horseradish peroxidase as described above.

### **Plasmid preparation**

#### **A. *E.coli* transformation:**

Two  $\mu$ l of each stock solution of SureSilencing™ shRNA plasmid (SABiosciences) were used to transform Max Efficiency DH5 $\alpha$ ™ competent cells

(Invitrogen). The siRNA sequences contained in these plasmids are shown in Table 3.1. After mixing gently, followed by incubation on ice for 5-30 min, the cells were heat-shocked for 30 sec at 42°C without shaking, after which 250 µl of Super Optimal broth with Catabolite repression (SOC) medium [2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 20mM MgSO<sub>4</sub>, ddH<sub>2</sub>O to 1000 mL, 20mM glucose] was added and the cells were incubated with shaking at 200 rpm at 37°C for 1 hr in a horizontal shaker (Hanahan, 1983). Ten to 50 µl from each transformation was spread on prewarmed LB (Luria-Bertani) plates containing 100 µg/ml ampicillin. Plates were incubated overnight at 37°C.

**Table 3.1: shRNA plasmid sequences used for silencing genes**

Product	Unigen e	Gen Bank	Symbol	Clone or Ex#	Sequence
KR44274	Rn.911 3	NM_0317 31	Aldh3a2	1	TCACTGATGTTGACCC TAACT
KR44274	Rn.911 3	NM_0317 31	Aldh3a2	2	GCAGCGGTTTGATCA CATTCT
KR44274	Rn.911 3	NM_0317 31	Aldh3a2	3	GGGAGAGAGTGTTAA CAAAC
KR44274	Rn.911 3	NM_0317 31	Aldh3a2	4	CTGTATTGCTCCTGAC TATAT
KR52689	Rn.878 21	NM_0536 23	Acsl4	1	GTAGAAGGATCTTGG GTTGAT
KR52689	Rn.878 21	NM_0536 23	Acsl4	2	GAAGGTGGTTATACAG TTCAT
KR52689	Rn.878 21	NM_0536 23	Acsl4	3	GGACACATATATTGGC TACTT
KR52689	Rn.878 21	NM_0536 23	Acsl4	4	TGACCATGCTGTAGC CAAGTT
KR44240	Rn.891 3	NM_0124 89	Acaa1	1	ATCTCTGTGGGTAAC GTACTT
KR44240	Rn.891 3	NM_0124 89	Acaa1	2	ATGGCGCGCATTGCC CAATTT
KR44240	Rn.891 3	NM_0124 89	Acaa1	3	AAAGCCAGAGACTGC CTGATT
KR44240	Rn.891 3	NM_0124 89	Acaa1	4	GTGGAAGTCCCTGT CAAGTA
Scrambled	RNAi	used	as	control	GGAATCTCATTGATG CATA



## **B. Plasmid purification:**

Approximately 10 colonies were picked from each plate with a sterile loop or toothpick and were used to inoculate separate 2.5 ml cultures of LB medium containing 100 µg/ml ampicillin, incubated with shaking at 37°C for 6 hrs until just a hint of turbidity was observed. Then these small cultures were used to inoculate 250 ml of LB medium containing 100 µg/ml ampicillin and incubated overnight. The cells were then collected and the plasmid DNA was purified using the Wizard Plus SV Midiprep DNA Purification System (Promega) for high copy number plasmids.

## **Transfections**

The day before transfection, McA-RH7777 hepatoma cells were plated in 6-well plates at a density of  $8 \times 10^4$  cells per  $\text{cm}^2$ . Plasmid DNA was transfected into cells with use of Fugene 6 transfection reagent (Roche Diagnostics), as follows: Plasmid DNA was mixed with Fugene 6 transfection reagent in serum-free medium at a ratio of 1 µg:5 µl and complexes were allowed to form for a minimum of 30 min at room temperature. The medium then was replaced with antibiotic-free medium with 10% FBS and the transfection mixture was added drop-wise to the cells with gentle swirling. Cells were assayed 24 hr post transfection for transfection efficiency (~25-30%) as determined by cotransfection of a β-galactosidase expression plasmid.

## **RT-PCR**

**A. RNA isolation:** Total RNA was isolated from cells with the use of TRIzol reagent (Invitrogen) and the concentration of RNA was measured spectrophotometrically with the use of a Nanodrop instrument (Thermo Scientific).

**B. Reverse transcriptase reaction:** First strand cDNA synthesis was performed using Superscript II Reverse Transcriptase (Invitrogen). Exactly 200

ng of random primers, 5 µg of total RNA, and 1 µl of dNTP mix (10 mM each nucleotide) were mixed in a total volume of 12 µl in diethylpyrocarbonate-treated water. The mixture was heated to 65°C for 5 min, quickly chilled on ice and then 4 µl of 5x First Strand Buffer, 2 µl of 0.1 M DTT, 1 µl RNaseOUT (Invitrogen) and 200 units of Superscript II Reverse Transcriptase was added. The reaction was incubated at 25°C for 10 min, followed by 50 min at 42°C and then inactivated by incubation at 70°C for 15 min. Synthesized single-stranded cDNA (sscDNA) was quantified by Nanodrop measurement (Thermo Scientific), aliquoted and stored at -20°C.

**C. Real-time PCR:** Gene-specific primers for real-time polymerase chain reaction (PCR) were designed using Primer 3 software (<http://frodo.wi.mit.edu/primer3/>). The sequences of the primers are shown in Table 3.2. Each 20-µl PCR reaction mixture contained 0.1 µg of single-stranded cDNA or template, 0.5 µl of gene-specific primers (10 µM each), 3 µl of dNTP mix (200 µM each nucleotide) and 1 x SYBR Green PCR buffer (Applied Biosystems). Cycling conditions were: 2 minutes at 50°C to activate, initial denaturation for 10 min at 95°C, followed by 50 cycles of denaturation for 15 sec at 95°C and 1 min of annealing/extension at 55°C. In order to detect nonspecific amplification, dissociation curves were established for each PCR product. The detection and quantitation of nucleic acids levels was done by using the comparative delta-delta ct method (Livak and Schmittgen, 2001).

**Table 3.2: Gene specific primers used in real-time PCR.**

<b>Gene</b>	<b>Primers</b>
<b><math>\beta</math> actin</b>	Fw: 5'-CCG TGA AAA GAT GAC CCA GAT C -3' Rw: 5'- AAT GCC AGT GGT ACG ACC AGA -3'
<b>ALDH3A2</b>	Fw: 5'- GCG AGA GAA GGA CAT CTT GG -3' Rw: 5'- TCG TCC ATC ATG GTA AGC AG -3'
<b>ACSL4</b>	Fw: 5'- CAC CAT TGC CAT TTT CTG TG -3' Rw: 5'- ATA ATG CCG CCT TCA GTT TG -3'
<b>ACAA1</b>	Fw: 5'- GGT CCA AGG CTG AAG AAC TG -3' Rw: 5'- CAG TAG AGG GCC TGA CTT GC -3'

## CHAPTER FOUR: RESULTS

### **Phosphorylation of AMP-kinase and HMG-CoA reductase after policosanol treatment**

Cholesterol synthesis is a highly regulated pathway and is subject to transcriptional, translational, and post-translational modulation (Espenshade and Hughes, 2007). Singh et al. (2006) showed that policosanol decreased cholesterol synthesis but did not directly inhibit HMG-CoA reductase, and incubation of hepatoma cells with policosanol did not affect HMG-CoA reductase protein levels. However, reductase *activity* was decreased by up to 55% in lysates prepared from these cells, suggesting that HMG-CoA reductase activity was down-regulated by policosanol treatment and that this was the mechanism by which policosanol decreases cholesterol synthesis. Because HMG-CoA reductase protein levels were not changed by policosanol treatment, I considered the possibility that policosanol inactivates HMG-CoA reductase by promoting its phosphorylation by one of three protein kinases known to inactivate HMG-CoA reductase (Beg et al., 1987). AMP-kinase is the principal kinase involved in the phosphorylation-mediated inactivation of HMG-CoA reductase (Carling et al, 1989) and is itself activated by phosphorylation by upstream kinases (Hardie, 2003; Towler and Hardie, 2007). To determine if policosanol might be down-regulating the activity of HMG-CoA reductase, the ability of policosanol to activate AMP kinase by promoting its phosphorylation was determined. Policosanol increased AMP-kinase phosphorylation in hepatoma cells by more than 2.5-fold after 3-hr treatment, with maximal stimulation occurring at 15-25  $\mu\text{g/ml}$  (Fig 4.1A). These increases were statistically significant and were comparable to the increases seen with AICAR and metformin, both of which are known to activate AMP kinase by phosphorylation and served as positive controls. No change in the level of total AMP kinase protein levels were observed over the 3-hr period of the experiment, despite a marked increase in the activity of this enzyme. These results are

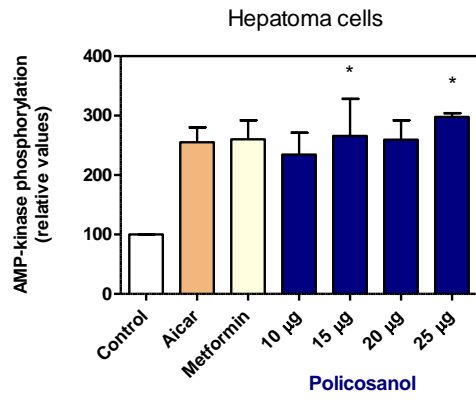
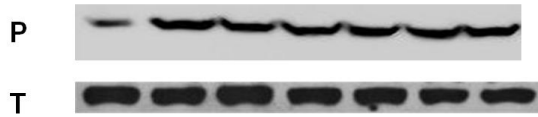
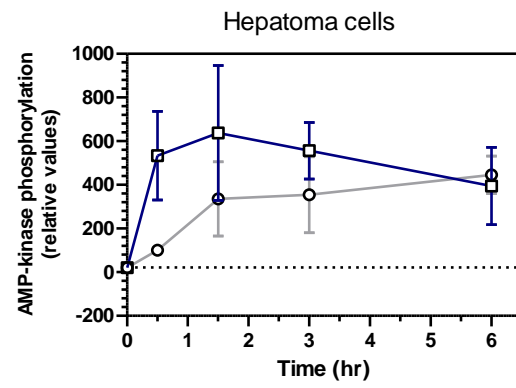
consistent with the *in vitro* studies of Singh et al. (2006), where it was shown that treatment of hepatoma cells with policosanol increased the phosphorylation of AMP-kinase and decreased HMG-CoA reductase activity and cholesterol synthesis.

Treatment of cells with policosanol rapidly activated AMP-kinase, with an increase in phosphorylation evident at 30 min; phosphorylation peaked at 1.5-hrs and stayed elevated through 3 hrs (Fig 4.1B). The activation of AMP-kinase by policosanol led to the concomitant phosphorylation of HMG-CoA reductase, as shown in Fig 4.2. Increases in HMG-CoA reductase phosphorylation occurred in a dose dependent manner with 10-25  $\mu\text{g/ml}$  of policosanol. Treatment of cells with AICAR or metformin yielded a similar increase in phosphorylated HMG-CoA reductase.

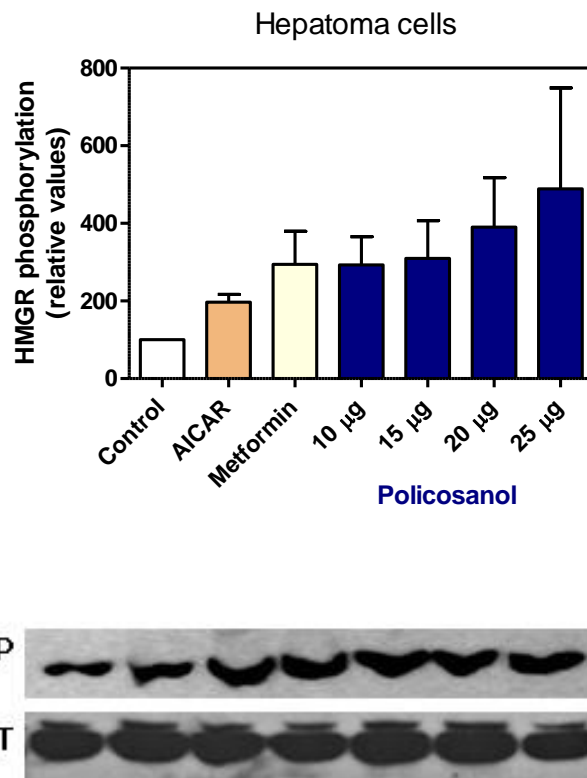
**Fig 4.1:** Policosanol activates AMP-kinase *in vitro*. (A) Phosphorylated AMP-kinase in hepatoma cells was measured by immunoquantitation with an antibody specific for the phosphorylated protein after a 3-h treatment with various concentrations of policosanol. The AMP-kinase activators AICAR and metformin (1 mM each) served as positive controls. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background. Values represent the mean and standard deviation of 3 experiments. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test,  $p < 0.05$ . Representative immunoblots of phosphorylated (P) and total (T) AMP-kinase are shown below the graph.

(B) Enzyme phosphorylation in hepatoma cells at various times after treatment with 15  $\mu\text{g/ml}$  of policosanol (*boxes*) or without policosanol (*circles*). Values represent the mean and standard deviation of four experiments; closed symbols are statistically different from untreated controls. Statistical significance was determined by one-way analysis of variance with Dunnett's *post hoc* text ( $P < .05$ ).

(B) Enzyme phosphorylation in hepatoma cells at various times after treatment with 15  $\mu\text{g/ml}$  of policosanol (*boxes*) or without policosanol (*circles*). Values represent the mean and standard deviation of three experiments; closed symbols are statistically different from untreated controls. Statistical significance was determined by one-way analysis of variance with Dunnett's *post hoc* text ( $P < .05$ ).

**A****B**

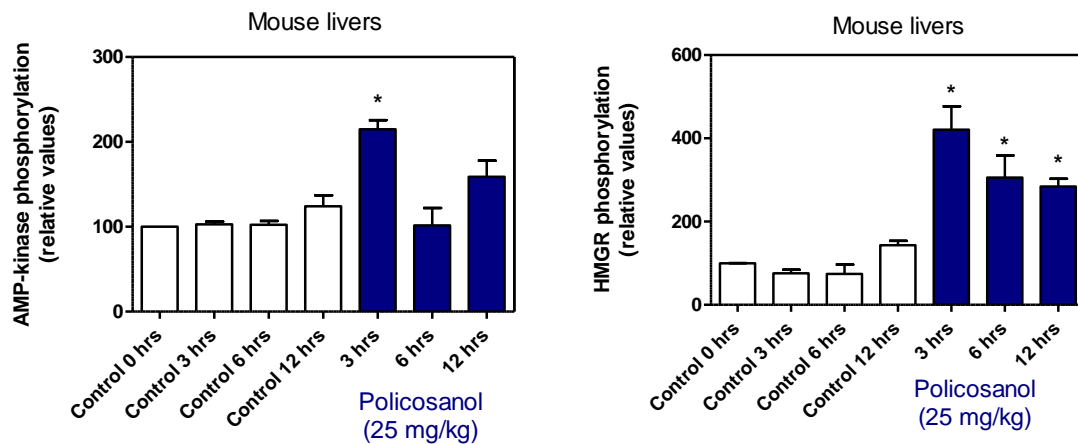
**Fig 4.2:** Policosanol inactivates HMG-CoA reductase *in vitro*. Phosphorylated HMG-CoA reductase in hepatoma cells was measured by immunoquantitation with an antibody specific for the phosphorylated protein after a 3-h treatment with various concentrations of policosanol. AICAR and metformin (1 mM each) served as positive controls. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background. Values represent the mean and standard deviation of 3 experiments. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test,  $p < 0.05$ . Representative immunoblots of phosphorylated (P) and total (T) HMG-CoA reductase are shown below the graph.



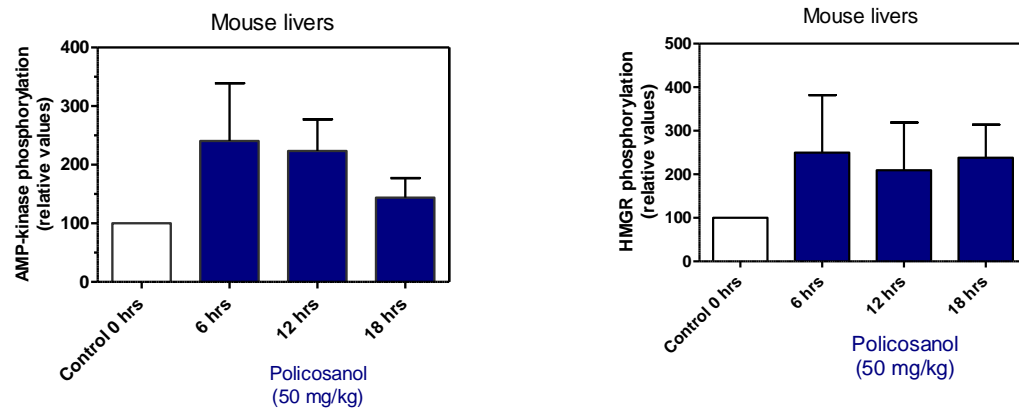


Menendez *et al.* (2005) found that free octacosanol and octacosanoic acid were present in the liver and plasma of rats fed policosanol, and that chain-shortened saturated (myristic, palmitic and stearic) and unsaturated (oleic, palmitoleic) fatty acids were formed after oral dosing of monkeys with policosanol. Moreover, because oral dosing is likely to result in significant hepatic exposure, the effective concentration therein is likely to be substantially higher; indeed, Kabir and Kimura (1995) reported that radiolabeled octacosanol is found predominantly in the liver after oral dosing. In studies by Menendez *et al.* (1996), oral administration of policosanol to rats decreased hepatic cholesterol biosynthesis while HMG-CoA reductase activity remained unchanged, suggesting that cholesterol synthesis was not inhibited by a direct action of policosanol on this enzyme. My data with cell culture preparations suggests that policosanol may promote the phosphorylation of HMG-CoA reductase by activation of AMP-kinase, and so it is reasonable to expect that it will act by the same mechanism *in vivo*. To confirm this supposition, the expression and activity of AMP-kinase and HMG-CoA reductase were assessed in the livers of mice gavaged with a single oral dose of policosanol at 10 mg/kg, 25 mg/kg, 50 mg/kg and 100 mg/kg. As shown in Figs 4.3, 4.4 and 4.5, policosanol was highly effective in promoting the phosphorylation of AMP-kinase as well as HMG-CoA reductase. AMP-kinase and HMG-CoA reductase phosphorylation increased by ~2 fold and ~4 fold respectively at 3 h with 25 mg/kg. Although at the 50 mg/kg dosing no 3-hr time point was taken, at 6 hr phosphorylation of both enzymes was increased by 2.5-fold. At 100 mg/kg dose phosphorylation was not increased, and in fact was significantly decreased for AMP-kinase at the 6-hr time point. Policosanol had no effect on AMP-kinase phosphorylation at 10 mg/kg dose (data not shown). Moreover no change in the total AMP-kinase was observed at each of time points and the dosing regimens tested.

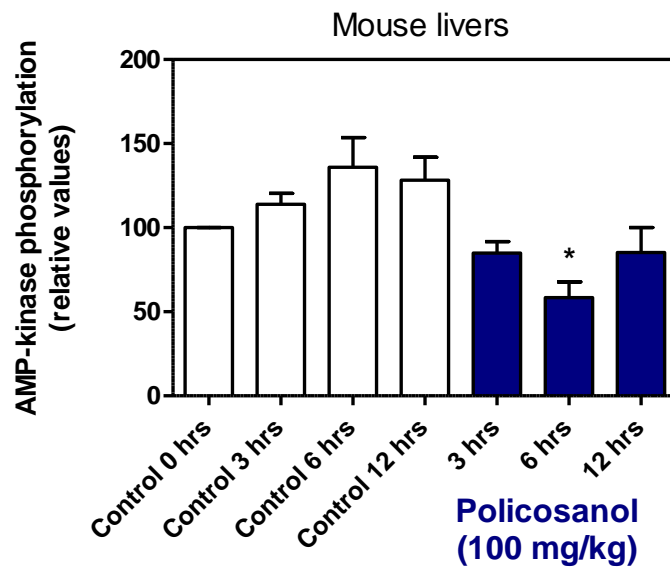
**Fig 4.3:** Single dose-response studies: Policosanol increases the phosphorylation of AMP-kinase and HMG-CoA reductase (HMGR) *in vivo* at 25 mg/kg. Phosphorylated AMP-kinase and HMG-CoA reductase were measured by immunoquantitation with antibodies specific for the phosphorylated proteins in mice gavaged with 25 mg/kg of policosanol. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background. Values represent the mean and standard deviation of 2-3 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test,  $p < 0.05$ .



**Fig 4.4:** Single dose-response studies: Policosanol increases the phosphorylation of AMP-kinase and HMG-CoA reductase *in vivo* at 50 mg/kg. Phosphorylated AMP-kinase and HMG-CoA reductase were measured by immunoquantitation with antibodies specific for the phosphorylated proteins in mice gavaged with 50 mg/kg of policosanol. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background. Values represent the mean and standard deviation of 2-3 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test,  $p < 0.05$ .



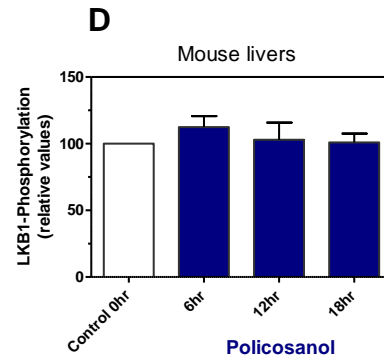
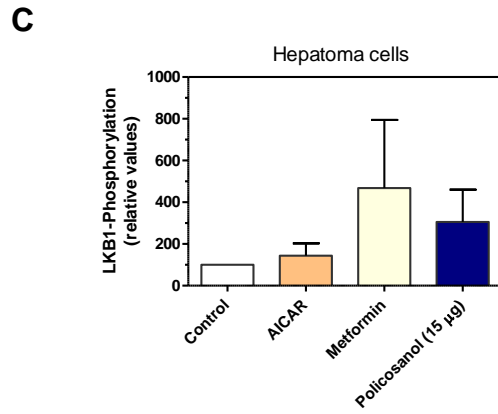
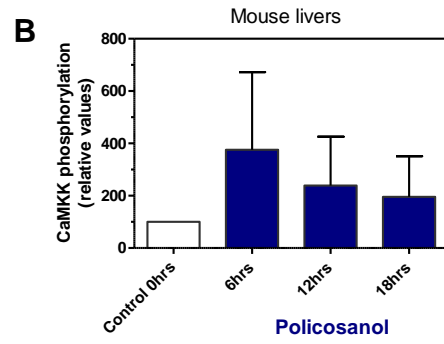
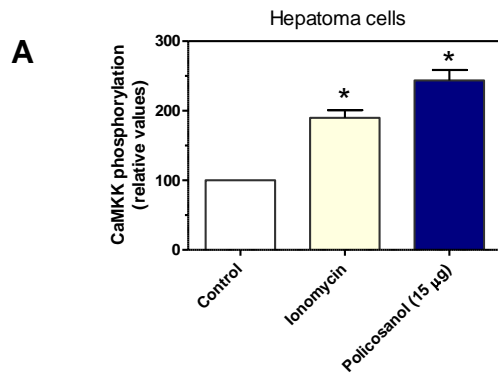
**Fig 4.5:** Single dose-response studies: Policosanol fails to increase the phosphorylation of AMP-kinase *in vivo* at 100 mg/kg. Phosphorylated AMP-kinase was measured by immunoquantitation with an antibody specific for the phosphorylated protein in mice gavaged with 100 mg/kg of policosanol. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background. Values represent the mean and standard deviation of 4 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test,  $p < 0.05$ . Because no increase in AMP-kinase phosphorylation was observed, HMG-CoA reductase phosphorylation was not tested.



## Activation of pathways upstream of AMP-kinase by policosanol

AMP-kinase itself is activated by phosphorylation by the upstream kinases LKB1 and TAK1; the binding of AMP to AMP-kinase makes the enzyme a better substrate for these kinases (Hardie, 2003; Towler and Hardie, 2007; Xie et al., 2009). In addition, three independent studies reported that CaMKK phosphorylates and activates AMP-kinase *in vitro*. Two of these studies provided evidence that CaMKK $\beta$ , rather than CaMKK $\alpha$ , is the predominant kinase acting on AMP-kinase. LKB1 is present in liver and muscle and is thought to be the principal activating enzyme for AMP-kinase in these tissues. Not surprisingly, LKB1 is also regulated by upstream kinases, most notably PKC $\zeta$  and perhaps also PKA and p90 RSK (Xie et al., 2008). As my studies have demonstrated that policosanol activates AMP-kinase both in cell culture and in mice *in vivo*, the upstream kinase pathways leading to AMP-kinase phosphorylation are an attractive route to explore in the effort to identify the mechanism by which policosanol activates this kinase. To identify the upstream kinase or kinases through which policosanol was signaling, I tested the effect of policosanol on CaMKK phosphorylation in hepatoma cells. The Ca<sup>2+</sup> ionophore, ionomycin served as a positive control. Fig 4.6A shows that CaMKK $\beta$  phosphorylation was increased by ionomycin and policosanol treatment ~ 2-fold and ~2.5 fold, respectively, relative to untreated cells. *In vivo*, policosanol treatment activated CaMKK $\beta$  by ~3.7-fold at 6 h and remained elevated till 18 h (Fig 4.6B). In comparison, when McA-RH7777 cells were incubated with policosanol, AICAR (negative control), or metformin (positive control) there was no significant increase in the LKB1 phosphorylation relative to control (Fig 4.6C). Similarly, no change in the amount of phosphorylation of LKB1 was observed in mice after gavaging with policosanol at 50 mg/kg dose (Fig 4.6D). No changes in the amount of phosphorylated PKC $\zeta/\lambda$  were noted either *in vitro* or *in vivo* with policosanol. These results indicate that although LKB1 is the principal AMP-kinase kinase, policosanol appears to utilize CaMKK $\beta$  to activate AMP-kinase.

**Fig 4.6:** Policosanol activates CaMKK but not LKB1. Phosphorylated CaMKK $\beta$  was measured by immunoquantitation with an antibody specific for the phosphorylated protein in (A) hepatoma cells after a 3-h treatment with policosanol (15  $\mu$ g/ml), or (B) *in vivo* at 6, 12, and 18 h after gastric gavage with 50 mg/kg body weight policosanol. Ionomycin served as a positive control in (A). Phosphorylated LKB1 was similarly immunoprecipitated from hepatoma cell lysates (C) or mouse liver homogenates (D) with an antibody to phosphoserine/phosphothreonine/phosphotyrosine and then quantified by immunoblotting with an antibody to total LKB1. Metformin (1 mM) served as a positive control and AICAR (1 mM) as a negative control in (A). Mice were gavaged with 50 mg/kg body weight of policosanol. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background. Values represent the mean and standard deviation of 2-3 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test,  $p < 0.05$ .



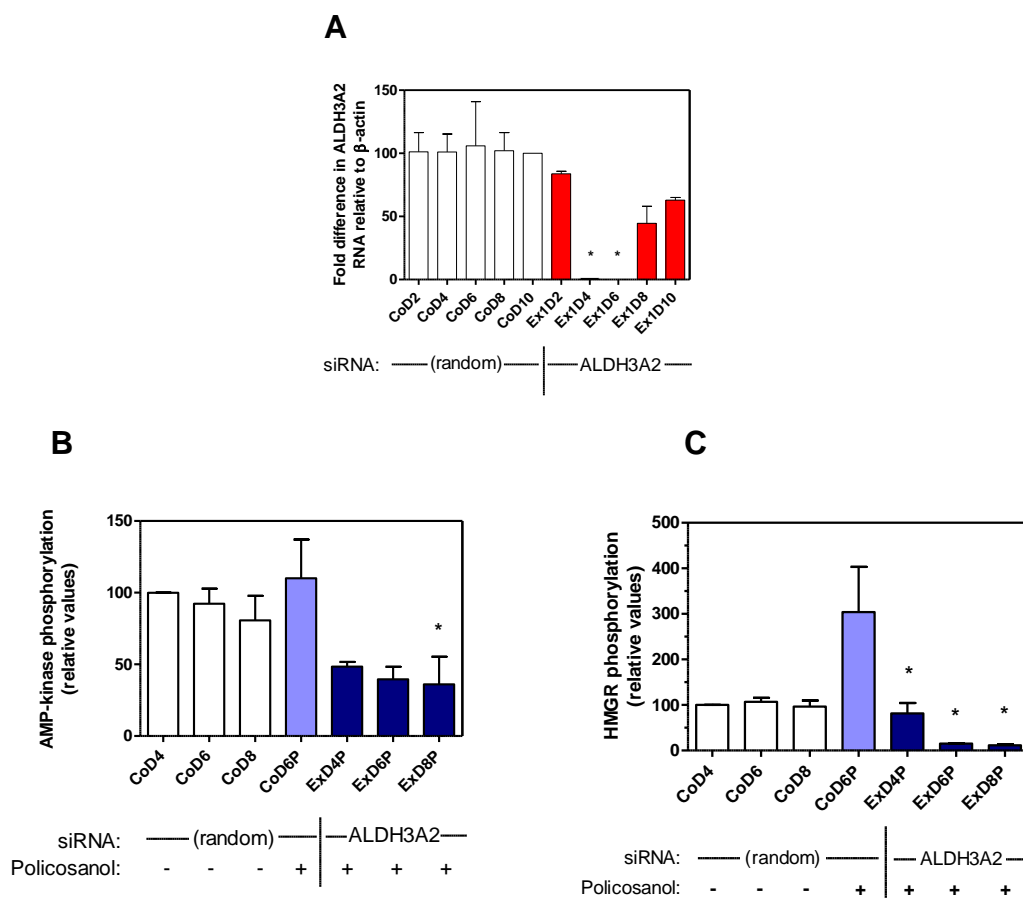
## **Activation of AMP-kinase by policosanol requires peroxisomal metabolism**

Regulation of fatty alcohol metabolism is poorly understood, and there is uncertainty as to whether the synthesis and oxidation of fatty alcohols is catalyzed by the same enzymes (Riendeau and Meighen, 1985). Long chain fatty alcohols are substrates for the 'fatty alcohol cycle' which converts long-chain alcohols to their corresponding fatty acids, and reduces long-chain fatty acids back to alcohols for incorporation into glycerol-ether lipids (Rizzo et al., 1987). These fatty acids perhaps undergo subsequent peroxisomal  $\beta$ -oxidation as was first revealed by the recognition that patients with inherited disorders of peroxisomal biogenesis had impaired ether lipid synthesis (Heymans et al., 1983; Heymans et al., 1984; Schutgens et al., 1984; Datta et al., 1984). Also, defective peroxisomal  $\beta$ -oxidation leads to accumulation of very-long-chain fatty acids (Rizzo, 1998). The fatty alcohol pathway is found on both the endoplasmic reticulum and peroxisomal surface (Rizzo, 1998; Hargrove et al., 2004). To determine if the metabolism of long-chain fatty alcohols that make up policosanol occurs through the fatty alcohol cycle, and if subsequent peroxisomal  $\beta$ -oxidation is necessary for activation of AMP-kinase, I suppressed the expression of 1) fatty aldehyde dehydrogenase 3A2 (ALDH3A2), 2) the peroxisomal acyl-CoA synthetase long-chain family member 4 (ACSL4), and 3) peroxisomal acetyl-coenzyme A acyltransferase 1 ( $\beta$ -keto thiolase, ACAA1) using RNA interference (RNAi) to decrease the mRNA levels for these enzymes. To ensure that the RNAi decreased the expression of these enzymes, mRNA levels for these enzymes were measured both in treated and scrambled RNAi-treated cells at different timepoints (2-10 days) by real-time polymerase chain reaction (PCR). The scrambled RNAi plasmid serves as a negative control for these studies. There was significantly greater than 95% suppression of fatty aldehyde dehydrogenase 3A2 expression at 4 and 6 days (Fig 4.7A), greater than 95% suppression of peroxisomal acyl-CoA synthetase 4 expression at 2, 4, 6 and 8 days (Fig. 4.8A), and greater than 80% and 95% suppression of peroxisomal acetyl-coenzyme A acyltransferase 1 expression at 4 and 6 days

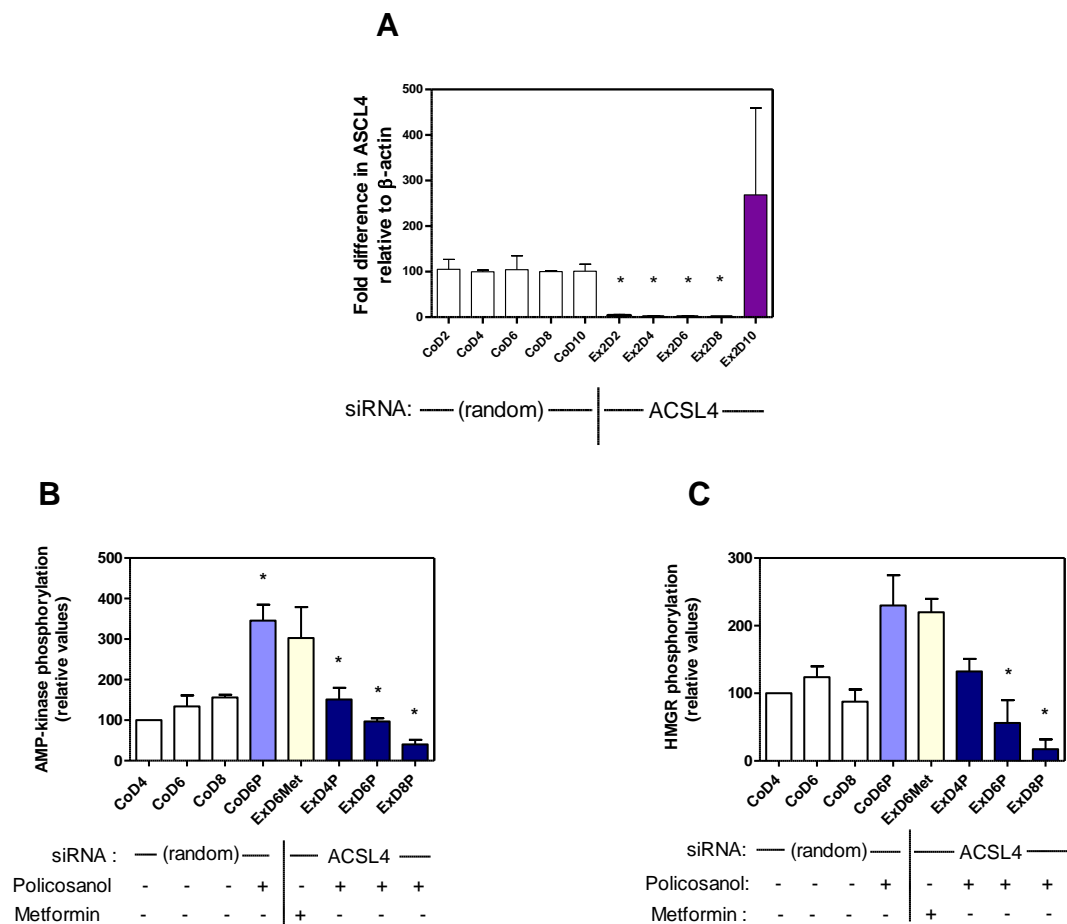


respectively (Fig. 4.9A). The suppression of these enzymes proved successful in preventing the increase in phosphorylation of AMP-kinase and HMG CoA-reductase after policosanol treatment. AMP-kinase phosphorylation was significantly decreased at 8 days (relative to the policosanol-treated 6-day control) in cells in which ALDH3A2 expression was suppressed (Fig 4.7B), and at 4, 6, and 8 days in cells in which ACSL4 expression was suppressed (Fig 4.8B). Suppression of ACAA1 yielded non-significant decreases in the phosphorylation of AMP-kinase relative to policosanol-treated control cells (Fig 4.9B). However, the increase in HMG-CoA reductase phosphorylation after policosanol treatment was significantly attenuated in cells in which ALDH3A2 (Fig 4.7C), ACSL4 (Fig 4.8C), and ACAA1 (Fig 4.9C), expression was suppressed. These results indicate that the production of either acetyl-CoA or propionyl-CoA formed after complete  $\beta$ -oxidation of policosanol (Fig 4.10) leads to the phosphorylation of HMG-CoA reductase by policosanol.

**Fig 4.7:** siRNA-mediated suppression of fatty aldehyde dehydrogenase 3A2 (ALDH3A2) prevents the increase in phosphorylation of AMP-kinase and HMG-CoA reductase by policosanol. Using the four individual shRNA plasmids supplied, one plasmid was selected that provided the greatest decrease in mRNA levels over a period of 2-10 days using the real-time qRT-PCR procedure (data not shown). Using that selected plasmid and control cells with a scrambled RNAi plasmid, AMP-kinase and HMG-CoA reductase phosphorylation was measured by immunoquantitation as described earlier after incubating the cultures with 15  $\mu\text{g/ml}$  policosanol. Values represent the mean and standard deviation of 2-3 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Tukey's post-hoc test,  $p < 0.05$ .

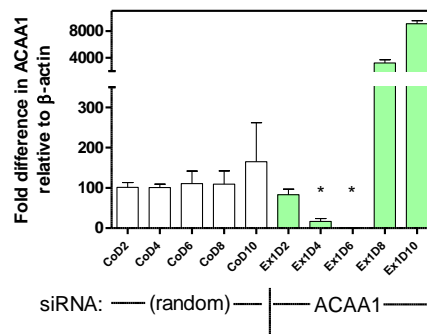


**Fig 4.8:** siRNA-mediated suppression of peroxisomal fatty acyl CoA synthetase-4 (ACSL4) prevents the increase in phosphorylation of AMP-kinase and HMG-CoA reductase by policosanol. Using the four individual shRNA plasmids supplied, one plasmid was selected that provided the greatest decrease in mRNA levels over a period of 2-10 days using the real-time qRT-PCR procedure (data not shown). Using that selected plasmid and control cells with a scrambled RNAi plasmid, AMP-kinase and HMG-CoA reductase phosphorylation was measured by immunoquantitation as described above after incubating the cultures with 15  $\mu$ g/ml policosanol or 1 mM metformin. Values represent the mean and standard deviation of 2 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Tukey's post-hoc test,  $p < 0.05$ .

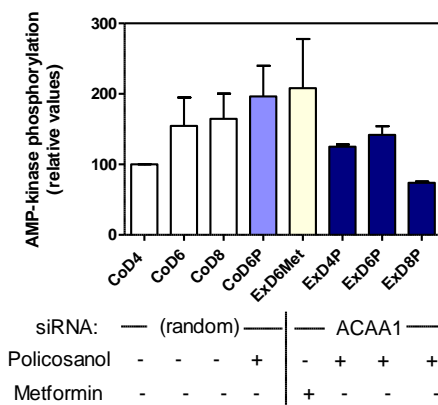


**Fig 4.9:** siRNA-mediated suppression of acetyl-coenzyme A acyltransferase 1 (ACAA1) prevents the increase in phosphorylation of HMG-CoA reductase by policosanol. Using the four individual shRNA plasmids supplied, one plasmid was selected that provided the greatest decrease in mRNA levels over a period of 2-10 days using the real-time qRT-PCR procedure (data not shown). Using that selected plasmid and control cells with a scrambled RNAi plasmid, AMP-kinase and HMG-CoA reductase phosphorylation was measured by immunoquantitation as described earlier after incubating the cultures with 15  $\mu$ g/ml policosanol or 1 mM metformin. Values represent the mean and standard deviation of 2 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Tukey's post-hoc test,  $p < 0.05$ .

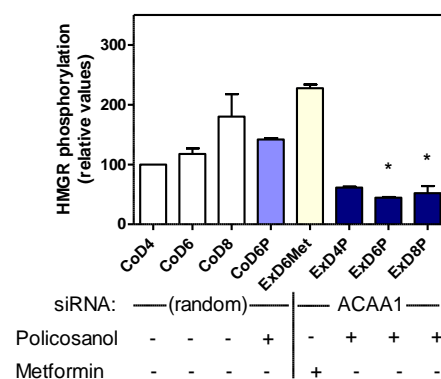
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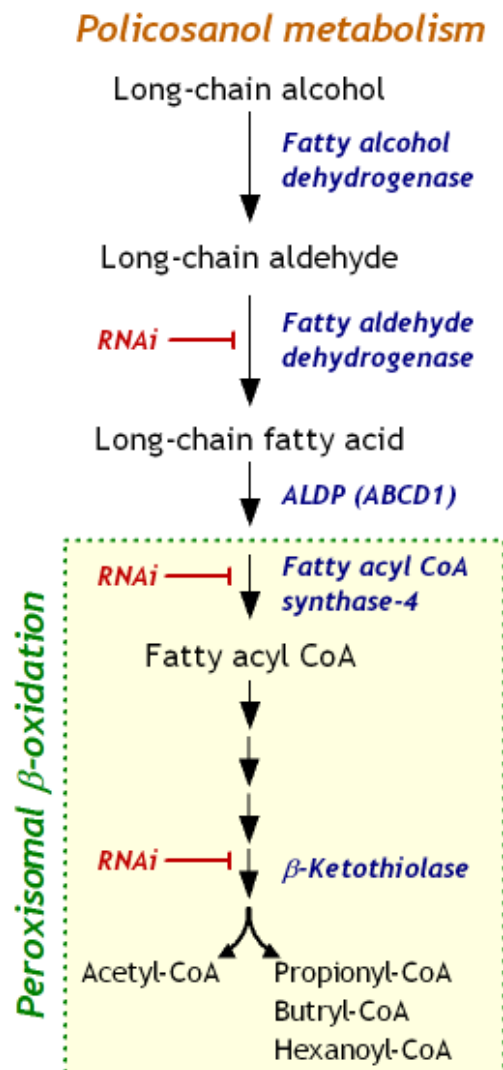
**B**



**C**



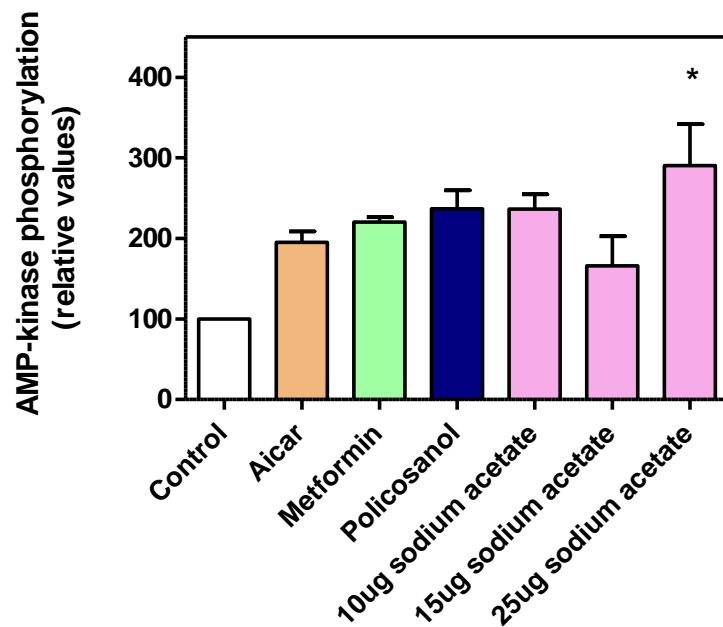
**Fig 4.10:** Schematic diagram of policosanol metabolism to acetyl-CoA and propionyl-CoA through the fatty alcohol cycle and peroxisomal  $\beta$ -oxidation. The enzymes which were suppressed by RNAi in the present experiments are indicated.



### **Activation of AMP-kinase by policosanol results from the generation of excess acetyl-CoA via peroxisomal metabolism**

Increased short-chain fatty acid production (including acetate) by colonic bacterial fermentation of dietary fibers has been reported to have several beneficial metabolic effects including lowering of plasma cholesterol (Higgins, 2004). It has also been suggested that activation of AMP-kinase in the liver might be the principal mechanism for the hypoglycemic effect of acetate (Sakakibara *et al.*, 2006). As the principal product of fatty acid  $\beta$ -oxidation is acetyl-CoA, it was of interest to see if addition of acetate to cells similarly promoted the phosphorylation of AMP-kinase and HMG-CoA reductase. Indeed, treatment of hepatoma cells with 10 to 25  $\mu$ g of sodium acetate for 3 hr yielded a statistically significant increase in the phosphorylation of AMP-kinase at the 25  $\mu$ g/ml dose (Fig. 4.11).

**Fig 4.11:** Treatment of hepatoma cells with sodium acetate promotes the phosphorylation of AMP-kinase. Phosphorylated AMP-kinase was measured by immunoquantitation with an antibody specific for the phosphorylated enzyme in hepatoma cells after a 3-h treatment with the AMP-kinase activators AICAR or metformin (1 mM each), policosanol (15  $\mu\text{g}/\text{ml}$ ) or 10-25  $\mu\text{g}/\text{ml}$  sodium acetate. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background. Values represent the mean and standard deviation of 3 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test,  $p < 0.05$



This result also is in agreement with several published studies: Kawaguchi *et al.* (2002) and Sakakibara *et al.* (2006) reported that acetate treatment of rat hepatocytes increased AMP-kinase and acetyl CoA carboxylase activation, the latter being a substrate for AMP-kinase. This increase in AMP-kinase activity appeared to result from an increase in AMP content of the hepatocytes (Kawaguchi *et al.*, 2002). The liver is responsible for most of the metabolism of acetate in the portal circulation (Mortensen *et al.*, 1996) where acetyl-CoA synthase catalyzes the following reaction:



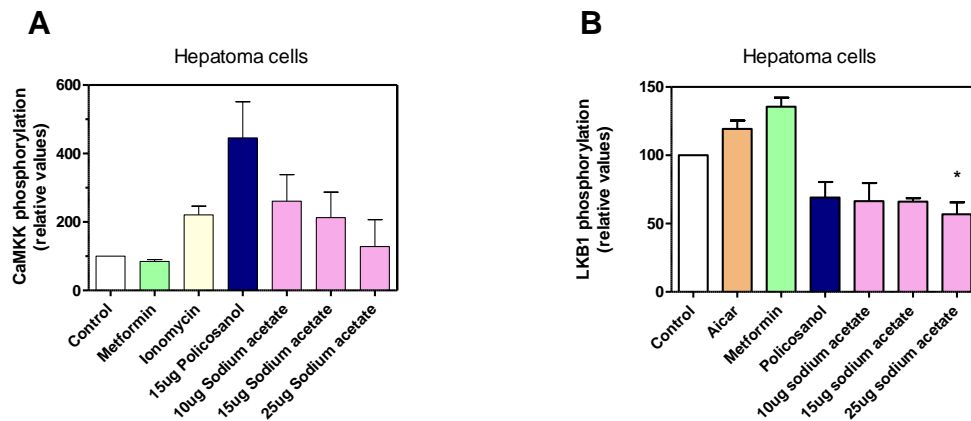
Zydowo *et al.* (1993) also reported increased hepatic AMP content in the livers of mice after intraperitoneal administration of acetate. As AMP promotes the phosphorylation of AMP-kinase, these studies suggest that acetate activation to acetyl-CoA promotes the phosphorylation of AMP-kinase through an increase in cellular AMP levels.

In recent years it has been determined that both the tumor-suppressor kinase LKB1 (Woods *et al.*, 2003; Hawley *et al.*, 2003; Shaw *et al.* , 2004) and CaMKK (Hurley *et al.*, 2005; Woods *et al.*, 2005; Hawley *et al.*, 2005) are important AMPK-kinases, which activate AMP-kinase by directly phosphorylating  $\alpha\text{Thr}^{172}$ . As my *in vitro* and *in vivo* studies with policosanol have shown that it acts through CaMKK $\beta$  and not through LKB1, and as acetyl-CoA or propionyl-CoA are the metabolites formed from policosanol, I wanted to determine if addition of sodium acetate to hepatoma cells would similarly promote the phosphorylation of CaMKK $\beta$  but not LKB1. As shown in Fig 4.12A, there was a more than 2-fold increase in the CaMKK $\beta$  phosphorylation in hepatoma cells with a low concentration (10-15  $\mu\text{g/ml}$ ) of sodium acetate. Although not statistically significant, this increase was comparable to the increase seen with ionomycin, a positive activator of CaMKK. However, similar to policosanol, there was a



dose-dependent decrease in LKB1 phosphorylation relative to control with sodium acetate at similar doses (Fig 4.12B). Thus, my studies indicate that sodium acetate, like policosanol, mediates activation of AMPK via CaMKK $\beta$ .

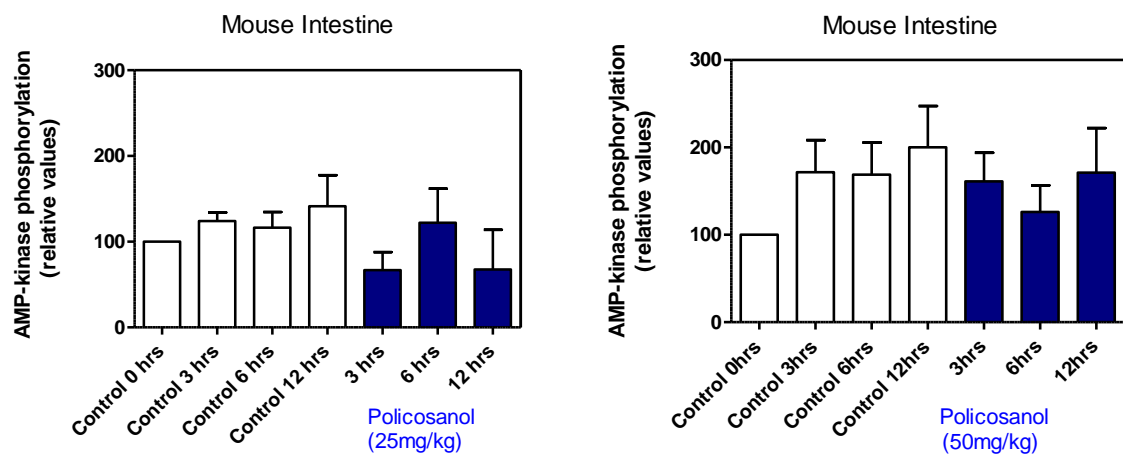
**Fig 4.12:** Sodium acetate treatment increases the phosphorylation of CaMKK but not LKB1. Phosphorylated CaMKK $\beta$  was measured by immunoquantitation with an antibody specific for the phosphorylated enzyme in hepatoma cells after a 3-h treatment with 1  $\mu$ g/ml ionomycin, 5  $\mu$ g/ml policosanol, or 10-25  $\mu$ g/ml sodium acetate. Phosphorylated LKB1 was immunoprecipitated from hepatoma cell lysates with an antibody to phosphoserine/phosphothreonine/phosphotyrosine after treatment of the cells with either AICAR or metformin (1 mM each), 15  $\mu$ g/ml policosanol, or 10-25  $\mu$ g/ml sodium acetate, and then quantified by immunoblotting with an antibody to LKB1. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background. Values represent the mean and standard deviation of 2-3 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test,  $p < 0.05$ .



### **Policosanol does not activate AMP-kinase in the small intestine**

As up to 25% of total serum cholesterol is obtained from intestinal synthesis, I wanted to determine the effect of oral administration of policosanol on the phosphorylation state of AMP-kinase in the small intestine. As shown in Fig 4.13, policosanol did not promote the phosphorylation of AMP-kinase in the small intestine of mice. Thus, it appears that policosanol would not affect cholesterol synthesis in the intestine through modulation of AMP-kinase.

**Fig 4.13:** Policosanol does not activate AMPK in mouse intestine. Phosphorylated AMP-kinase was measured by immunoquantitation-kinase with an antibody specific for the phosphorylated enzyme in the proximal intestine of mice gavaged with 25 mg/kg or 50 mg/kg of policosanol. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background. Values represent the mean and standard deviation of 3-4 experiments; values that are statistically different from untreated controls are indicated with *asterisks*. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test,  $p < 0.05$ .



## CHAPTER FIVE: SUMMARY OF RESULTS

Policosanol is a mixture of very long chain alcohols isolated from sugarcane and other plant-derived sources that has been reported to decrease serum cholesterol in animals and humans. In a number of short as well as long term clinical studies policosanol was successful in lowering LDL cholesterol by 33% in normocholesterolemic patients and 24% in hypercholesterolemic patients (Gouni-Berthold and Berthold, 2002; Varady et al., 2003). In these patients policosanol also increased HDL cholesterol by an average of 17% without affecting triglyceride levels. However, little scientific evidence of the mechanism by which policosanol lowers blood cholesterol is known. Previous studies have reported that policosanol at pharmacological concentrations acted at or above HMG-CoA reductase (Singh et al., 2006), the third enzyme and the regulatory step in the cholesterol synthesis pathway, to decrease cholesterol synthesis. Further studies demonstrated that policosanol did not act as a direct inhibitor of this enzyme and did not affect HMG-CoA reductase enzyme levels (Singh et al., 2006). As reductase activity was decreased by up to 55% without a change in enzyme levels, Singh et al. (2006) suggested that HMG-CoA reductase was being inactivated by phosphorylation by AMP-kinase. My studies extended and confirmed this hypothesis by showing that policosanol increased the amount of phosphorylated AMP-kinase in cells by more than 2.5 to 3-fold after 3 h of treatment at 15- 25 µg/ml, and concurrently increased the phosphorylation of HMG-CoA reductase in a dose dependent manner. The time course of activation was also rapid with policosanol, reaching a peak at relatively shorter period of 1.5 hrs.

As several studies in animal models have indicated that oral administration of policosanol decreases cholesterol synthesis *in vivo* (Arruzazabala et al., 1994; Rodriguez-Echenique et al., 1994; Menendez et al., 1997), I also tested the ability of policosanol, administered by gavage, to activate AMP-kinase *in vivo*. The expression and activity of AMP-kinase and HMG-CoA reductase were

assessed in the livers of mice gavaged with a single oral dose of policosanol at 25 mg/kg, 50 mg/kg and 100 mg/kg. I observed a robust increase in AMP-kinase and HMG-CoA reductase phosphorylation at doses of 25 mg/kg and 50 mg/kg. AMP-kinase and HMG-CoA reductase phosphorylation increased by ~2 fold and ~4 fold respectively at 3 h with 25 mg/kg. Although at the 50 mg/kg dose no 3-hr time point was taken, at 6 hr phosphorylation of both enzymes was increased by 2.5-fold. At the 100 mg/kg dose phosphorylation was not increased, and in fact was statistically decreased for AMP-kinase at the 6-hr time point. No change in the total AMP-kinase was observed at each of time points and the dosing regimens tested. These results demonstrate that policosanol acts both *in vitro* and *in vivo* to activate AMP-kinase and to inactivate HMG-CoA reductase.

I also investigated the mechanism by which policosanol activates AMP-kinase. LKB1 and CaMKK $\beta$  are two upstream kinases that have been reported to activate AMP-kinase. LKB1 appears to be most important in liver (Towler and Hardie, 2007) while CaMKK $\beta$  is predominantly expressed in neural tissue, although much lower levels are detected in a variety of other tissues including the liver (Birnbaum, 2005). My studies have revealed that policosanol acts through CaMKK $\beta$  and not through LKB1 to activate AMP-kinase in the liver and in hepatoma cells.

It has been reported that D-003, a mixture of very long chain fatty acids, also suppresses HMG-CoA reductase activity (Menendez et al., 2001b) and that this effect was more robust than that of policosanol and occurred at a much faster rate. This observation suggested that the long chain primary alcohols of policosanol must undergo oxidation to the corresponding fatty acid in order to activate AMP-kinase; it raised the possibility that peroxisomal  $\beta$ -oxidation is also required. My studies show that siRNA-mediated suppression of fatty aldehyde dehydrogenase 3A2, fatty acyl CoA synthase 4, and acetyl-coenzyme A acyltransferase 1 in cell culture prevented the phosphorylation of AMP-kinase

and HMG-CoA reductase, indicating that metabolism of policosanol through the fatty alcohol cycle and subsequent peroxisomal  $\beta$ -oxidation is necessary for the activation of AMP-kinase.

Although the intestine is also a significant source of circulating cholesterol, my studies with policosanol did not reveal any measurable change in the activation of AMP-kinase in this tissue. One possible explanation for this lack of effect is that enterocytes may not metabolize policosanol through the fatty alcohol cycle and peroxisomal oxidation, as they are responsible for the absorption of lipids and their transfer into the bloodstream. Alternatively, it is possible that policosanol is not actually absorbed in the small intestine, but instead is metabolized to very short chain fatty acids, including acetate, by colonic bacteria, and that these substances are then absorbed in the distal colon, where they circulate to the liver to activate hepatic AMP-kinase (Wong et al., 2006). My studies are not able to distinguish between these two possibilities.

As the principal product of fatty acid  $\beta$ -oxidation is acetyl-CoA, my studies further demonstrated that addition of acetate to cells results in the phosphorylation of AMP-kinase and HMG-CoA reductase. This finding argues that the activation of AMP-kinase by policosanol results from the generation of excess acetyl-CoA via peroxisomal metabolism. These findings open new perspectives not just for the treatment of hypercholesterolemia, but may open new vistas for the control of diabetes and metabolic syndrome by activators of AMP-kinase.

## CHAPTER SIX: DISCUSSION

A sizeable amount of data from various animal studies and human clinical studies has suggested that policosanol may be an effective means to lower serum cholesterol levels. Low density lipoprotein (LDL) and total cholesterol reductions have been found in both normocholesterolemic and hypercholesterolemic patients (Gouni-Berthold and Berthold, 2002; Varady et al., 2003; Castano et al., 2005) after treatment with 5-20 mg of policosanol in both short-term ( $\leq 12$ -week) and long-term (up to 2-year) randomized, placebo-controlled, double-blind studies. However, several recent studies have been unable to demonstrate a significant reduction in serum cholesterol levels with policosanol treatment, calling into question the efficacy of this regimen (Kassis et al., 2009; McGowan and Proulx, 2009). Although few studies have investigated the mechanism underlying the actions of policosanol, it was speculated that the mixture is able to inhibit cholesterol synthesis and increase LDL clearance (Varady et al., 2003). In the present studies I show that policosanol is able to promote HMG-CoA reductase phosphorylation by activation of AMP-kinase. The increased phosphorylation of AMP-kinase can affect HMG-CoA reductase activity either by direct phosphorylation and consequent inactivation of the enzyme (Beg et al., 1978), or by an indirect down-regulation mediated by transcription factors such as SREBP-1 (Motoshima et al., 2006), thus suppressing cholesterol synthesis. In my studies, both the *in vitro* incubation of cultured McA-RH7777 hepatoma cells and the *in vivo* treatment of mice with policosanol stimulated the phosphorylation of both HMG-CoA reductase and AMP-kinase. AMP-kinase also inactivates acetyl-CoA carboxylase (Carling et al., 1987) and thus there is coordinate down-regulation of cholesterol and fatty acid biosynthesis by this kinase pathway.

Complicated interrelationships exist between the different kinases that activate AMP-kinase. LKB1, PPAR $\gamma$ , PKC $\zeta$  and CaMKK $\beta$  all have been suggested to



be involved in the activation of AMP-kinase. Although LKB1 is considered to be the major AMP-kinase, my studies both *in vivo* as well as *in vitro* show CaMKK $\beta$  to be the upstream AMPK-kinase through which policosanol acts. Consistent with my studies, CaMKK $\beta$  has also been shown to be required in receptor-modulated AMPK phosphorylation stimulated by simvastatin (Kou et al., 2009), a drug widely prescribed for the prevention and treatment of hypercholesterolemia and cardiovascular diseases.

D-003, a mixture of very long-chain fatty acids, inhibits cholesterol synthesis in cultured cells (Menendez et al., 2001b), and has been suggested to be more effective than policosanol in lowering blood cholesterol levels (Menendez et al., 2004). This greater effectiveness may be due to its greater proportion of fatty acids greater than 30 carbons in length; Singh *et al.* (2006) found that triacontanol, a C-30 alcohol, was the most effective component tested of the several C-26 to C-30 alcohols in policosanol. However, as my studies have demonstrated, policosanol must undergo oxidation, both through the fatty alcohol cycle (Rizzo et al., 1987) and via peroxisomal  $\beta$ -oxidation, to activate AMP-kinase. Thus, D-003, as a very long chain fatty acid mixture, may be more efficacious simply because it bypasses the fatty alcohol cycle and can directly enter the peroxisomal oxidation pathway. Because D-003 also suppresses HMG-CoA reductase activity (Menendez et al., 2001b), prior to my studies it was unclear if the very long chain alcohols in policosanol must first undergo oxidative metabolism via the fatty alcohol cycle to the corresponding fatty acids in order to be effective. It was also not known if subsequent peroxisomal  $\beta$ -oxidation occurred, and was required for the activation of AMP-kinase. Pharmacokinetic studies on octacosanol metabolism indicated that this very long-chain alcohol can undergo oxidation to CO<sub>2</sub> *in vivo*, presumably via these pathways (Kabir and Kimura, 1993). In the present study, siRNA-mediated suppression of several of the enzymes in these oxidative pathways was used to determine if the metabolism of the long-chain alcohols in policosanol via these pathways is required for the activation of AMP-kinase.

Using these siRNA-generating plasmids, selective suppression of the expression of three key enzymes, viz, fatty aldehyde dehydrogenase, fatty acyl CoA synthase 4, and acetyl-coenzyme A acyltransferase 1 ( $\beta$ -ketothiolase) in the fatty alcohol and  $\beta$ -oxidation pathways was carried out, and it was shown that both the fatty alcohol pathway and peroxisomal  $\beta$ -oxidation is required for the activation of AMP-kinase by policosanol. As it has been known for some time that very long chain fatty acids (VLCFA) inhibit cholesterol synthesis in cell culture (Murthy et al., 1988; Menendez et al., 2001a; Garcia-Pelayo et al., 2003), it is likely that peroxisomal oxidation is also the mechanism by which these lipids, as well as D-003, inhibit cholesterol synthesis.

Recently it was reported that acetic acid, when fed via a stomach tube, activates hepatic AMP-kinase and reduces hyperglycemia in diabetic mice (Sakakibara et al., 2006), and that it also improves insulin sensitivity in subjects with insulin resistance or type II diabetes (Johnston et al., 2004). My studies, in which cultured hepatoma cells were treated with acetate, also found increased phosphorylation of AMP-kinase. This finding, in conjunction with acetate feeding studies, suggests that acetate, generated from  $\beta$ -oxidation of the very long chain fatty acids formed from policosanol, is ultimately responsible for the activation of AMP-kinase. *In vivo*, all acetate taken up from the portal circulation is activated by the liver (Mortensen et al., 1996) to acetyl-CoA in a reaction that requires ATP. This may lead to depletion of ATP with the concomitant generation of AMP, an allosteric activator of AMP-kinase, when acetate is abundant in the portal bloodstream. Thus, the increased AMP-kinase activity in cells treated with acetate might be promoted by the increased AMP generated by acetate activation. Finally, Sakakibara et al. (2006) reported that sodium acetate directly activated AMP-kinase and lowered the expression of genes including sterol regulatory element binding protein-1 in rat hepatocytes, which might explain indirect downregulation of HMG-CoA reductase.

It is of interest that acetyl-CoA, produced through the  $\beta$ -oxidation of policosanol, is the required substrate for cholesterol biosynthesis, and thus an abundance of this substrate might be expected to increase cholesterol synthesis. A possible explanation for inhibition in cholesterol synthesis in the face of this substrate surplus could be that the phosphorylation of HMG-CoA reductase at Ser 872 by AMP-kinase causes a conformational change in the three dimensional shape of the enzyme that reduces the  $V_{max}$  but not the  $K_m$  of the reductase. In this sense, this conformational change resembles the effect of a reversible noncompetitive inhibitor. Noncompetitive inhibitors cannot be overcome even at high substrate concentrations (Lehninger et al., 2008).

Acetyl-CoA carboxylase (ACC) catalyzes the synthesis of malonyl-CoA, the first and committed step in fatty acid synthesis. ACC activity is also suppressed, like HMG-CoA reductase, by reversible phosphorylation by AMP-kinase. Phosphorylation on the  $\alpha$  subunit (ACC- $\alpha$ ) decreases the synthesis of fatty acids, while phosphorylation on the  $\beta$  subunit (ACC- $\beta$ ) leads to a decrease in malonyl-CoA levels and, moreover, a disinhibition of fatty acid oxidation (Munday and Hemingway, 1999). As my studies suggest that policosanol administration may lead to increased  $\beta$ -oxidation of fatty acids, it is reasonable to assume that concurrent fatty acid synthesis would represent a futile cycle for the cell. Thus, the activation of AMP-kinase, leading to the suppression of the regulatory enzyme in fatty acid synthesis, makes sense, as it would suppress fatty acid synthesis when fatty acids are abundant and available for oxidation. Similar to the effect of fibrates, the partitioning of fatty acids towards oxidation and away from esterification might be an important aspect of the lipid-lowering effects of policosanol.

As policosanol gavage did not affect AMP-kinase activation or HMG-CoA reductase phosphorylation in the small intestine, it leaves some unanswered questions: Is policosanol absorbed in the small intestine? Is the small intestine resistant to activation of AMP-kinase by this substance? Alternatively,

it is possible that policosanol is being metabolized to short chain fatty acids, including acetate, in the distal colon by the colonic bacteria, and these short-chain fatty acids are then absorbed and subsequently taken up by the liver (Wong et al., 2006).

The observation that policosanol activates AMP-kinase is reminiscent of the mechanism of metformin, a drug widely used to treat type II diabetes. Metformin not only reduces hyperglycemia but also improves insulin sensitivity and modestly lowers blood lipid levels (Zhou et al., 2001). Metformin acts through AMP-kinase to reduce insulin resistance, hyperinsulinemia and obesity (Zhou et al., 2001) and in many cases the actions of metformin can be mimicked by the AMP-kinase activator AICAR. However, metformin, unlike policosanol, acts through LKB1 to activate AMP-kinase (Shaw et al., 2005).

The suppression of HMG-CoA reductase, besides affecting cholesterol biosynthesis, could influence a variety of other physiological pathways, including cell cycle progression, as a consequence of the decreased synthesis of nonsterol mevalonate derivatives viz, farnesyl, geranyl and geranyl-geranyl intermediates. These isoprenoids are necessary for prenylation of Ras and Rho proteins involved in cell signaling that control cell proliferation. The present studies could help us discover the role that policosanol might play in regulating metabolic diseases like type II diabetes through pharmacological regulation of AMP-kinase.

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## VITA

### PERSONAL INFORMATION

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### EDUCATION

2004- 2010

Graduate Center for Toxicology,  
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2000- 2002

M.Sc in Zoology,  
University of Kalyani, West Bengal, India

1997-2000

BSc in Zoology,  
University of Calcutta, West Bengal, India

### WORK EXPERIENCE

2002-2003                      Researcher in Department of Hematology, NRSMCH  
Kolkata, India

## UNIVERSITY SERVICE

*University of Kentucky*

2007-2009      Resident Council, Graduate and Family Housing,  
University of Kentucky, Lexington Vice Chair

## HONORS AND AWARDS

2004- 2005      Research Challenge Trust Fund Scholarship,  
University of Kentucky

2007              SOT graduate student travel award at the annual SOT  
conference in Charlotte, North Carolina

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## PUBLICATIONS

Banerjee S, Ghoshal S and Porter TD. **Activation of AMP-kinase by policosanol requires peroxisomal metabolism** (submitted)

Banerjee S, Stolarczyk E and Porter TD. **Suppression of cytochrome P450 reductase expression in hepatoma cells replicates the hepatic lipidosis**

**observed in hepatic reductase-null mice** (in preparation)

Singh DK, Banerjee S, Porter TD. **Green and black tea extracts inhibit HMG-CoA reductase and activate AMP kinase to decrease cholesterol synthesis in hepatoma cells.** Journal of Nutritional Biochemistry (2008) PMID: 18926682

Glauert HP, Tharappel JC, Banerjee S, Chan NL, Kania-Korwel I, Lehmler HJ, Lee EY, Robertson LW, Spear BT. **Inhibition of the promotion of hepatocarcinogenesis by 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153) by the deletion of the p50 subunit of NF-kappaB in mice.** Toxicology and Applied Pharmacology (2008) PMID: 18644402

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## **PRESENTATIONS**

Todd D Porter, Subhashis Banerjee, and Elzbieta I Stolarczyk (2010) **Suppression of cytochrome P450 reductase expression in hepatoma cells replicates the hepatic lipidosis observed in hepatic reductase-null mice.** Annual Experimental Biology meeting, Anaheim, CA.

Subhashis Banerjee and Todd D Porter (2010) **Tea and policosanol act through different mechanisms to activate AMP-kinase and suppress HMG-CoA reductase to inhibit cholesterol synthesis.** Annual Experimental Biology meeting, Anaheim, CA,

Subhashis Banerjee and Todd D. Porter (2009) **Activation of AMP-kinase and suppression of HMG-CoA reductase by policosanol requires peroxisomal metabolism.** Gill Heart Cardiovascular Research Conference, Lexington, KY

Elzbieta Stolarczyk, Subhashis Banerjee and Todd D. Porter (2009) **Suppression of cytochrome P450 reductase expression in hepatoma cells replicates the hepatic lipidosis observed in hepatic reductase-null mice** Gill Heart Cardiovascular Research Conference, Lexington, KY.

Daniel Katz , Subhashis Banerjee and Todd Porter (2009) **Activation of AMP-Kinase in Mouse Liver Following Oral Administration of Policosanol** Summer Undergraduate Research Program Poster Session University of Kentucky, Lexington.

Subhashis Banerjee and Todd D. Porter (2009) **Inhibition of cholesterol synthesis by tea and policosanol: Identification of the pathways by which these botanicals activate AMP-kinase** 50<sup>th</sup> anniversary meeting of the American Society of Pharmacognosy, Honolulu, Hawaii and The Gordon Research Conference on Molecular and Cellular Biology of Lipids, Waterville Valley, NH.

Subhashis Banerjee, Dev K Singh and Todd Porter (2008) **Mechanisms underlying the hypocholesterolemic effects of green and black tea extracts** Gill Heart Cardiovascular Research Conference, Lexington, KY 2008.

Melissa Douglas, Subhashis Banerjee, Elzbieta Stolarczyk, Todd Porter (2008) **Small Interfering RNA-Mediated Silencing of Cytochrome P450 Reductase**



**in Rat Hepatoma Cells** Summer Undergraduate Research Program Poster Session University of Kentucky, Lexington.

Subhashis Banerjee, Lap Shun Chan, Job C. Tharappel, Brett T. Spear, Hans-Joachim Lehmler, Larry W. Robertson and Howard P. Glauert (2007)

**Alteration of growth regulation during the promotion of hepatocarcinogenesis by PCBs in mice lacking the p50 subunit of NF- $\kappa$ B**

Annual Meeting of the Society of Toxicology, Charlotte, NC.