UNIVERSITY OF ALBERTA

# The absorption and metabolism of policosanol in Caco-2 cells —a potential natural cholesterol-lowering agent

By

Qun Li ©

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# ABSTRACT

Policosanol is a mixture of very long chain fatty alcohols, that has been studied in the last decade for cholesterol-lowering. This study was conducted to examine absorption and metabolism of policosanol by Caco-2 cells monolayer.

Expressed as disappearance, the absorption rate of policosanol is 86.0% after 48 hours incubation.

Policosanol significantly increased the amount of cholesterol ester fatty acid both in the cells and lymphatic medium, while, significantly decreasing the cholesterol ester fatty acid amount in the luminal medium. Policosanol also significantly increased the free fatty acids amount in the lymphatic medium, while significantly decreasing the free fatty acids amount in the cells. Further evaluation of lipid profiles indicated that policosanol significantly increased the n-6 PUFA of cholesterol ester in the lymphatic medium. Policosanol significantly increased the proportion of behenic acid (C22). It is concluded that policosanol or policosanol metabolites will modulate lipid metabolism or/and transport after being absorbed.

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## ABBREVIATIONS

| | |
|---|---|
| AA | arachidonic acid |
| ACAT | acyl-CoA: cholesterol acyltransferase |
| apoA | apolipoprotein A |
| apoB | apolipoprotein B |
| BCA | bicinchoninic acid |
| BSA | Bovine Serum Albumin |
| BW | Body Weight |
| CD | combined dyslipidemia |
| CHD | coronary heart disease |
| CVD | cardiovascular disease |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| EDTA | ethylenediaminetetraacetic acid |
| EMEM | Earle's Minimum Essential Medium |
| FALDH | fatty aldehyde dehydrogenase |
| FAO | fatty alcohol:NAD1 oxidoreductase |
| FBS | fetal bovine serum |
| FID | flame ionization detector |
| GC | gas chromatography |
| GLC | gas-liquid chromatograph |
| Hb | hemoglobin |
| HC | patients with hypercholesterolemia type II. |
| HDAP | alkyl dihydroxyacetone phosphate |
| HDL-C | high-density lipoprotein cholesterol |
| HMG-CoA | 3-hydroxy-3-methyl-glutaryl-CoA |
| HV | healthy volunteer |
| LDL | low-density lipoprotein |
| LDL-C | low-density lipoprotein cholesterol |
| M | microvilli |
| MSTFA | N-methyl-N-(trimethylsilyl) trifluoracetamide |

| | |
|---|---|
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NS | not significant |
| NIDDM | non-insulin-dependent diabetes mellitus |
| PBS | phosphate buffered saline |
| PC | phosphatidyl choline |
| RCDP | Rhizomelic Chondrodysplasia Punctata |
| SEM | scanning electron microscopy |
| SFP | sunflower oil production |
| SMC | smooth muscle cell |
| TC | total cholesterol |
| TEER | transepithelial electric resistance |
| TEM | transmission electron microscopy |
| TLC | Thin-layer Chromatography |
| TJ | tight junctions |
| TMS | trimethlsilyl |
| VLCFA | very long chain fatty acids |
| WGP | wheat germ policosanol |

# Chapter 1

# INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of death in many developed countries as well as in some developing countries. High blood cholesterol level is the major risk contributing to CVD. In recent years, many natural compounds have attracted much interest due to potential as functional neutraceuticals to treat hypercholesterolemia. Policosanol refers to a group of very long chain primary alchohols (C24-34), that is usually isolated from sugarcane wax, rice bran or beeswax, by solvent extraction and saponification. Policosanol has been shown to lower plasma total cholesterol (TC) and low-density lipoprotein (LDL) cholesterol and elevate high-density lipoprotein cholesterol (HDL-C) in hypercholesterolemia patients (Castano et al, 2000; Pons et al, 1992), in type 2 diabetics (Castano et al, 2002) and in healthy volunteers (Hernandez et al, 1992). A favorable effect of policosanol in studies conducted mainly on plasma lipids has been demonstrated in various animal models studies conducted in Cuba. Policosanol also has been demonstrated to inhibit LDL oxidation (Menendez et al, 1999) and decrease platelet aggregation and production of thromoboxane B2 (Carbajal et al, 1998; Arruzazabala et al, 1993).

Recently, all potential benefits associated with policosanol have not always been observed in other studies (Wang et al, 2003; Lin, et al, 2004; Berthold et al, 2006). The mechanism by which policosanol reduces plasma TG remains poorly understood.

## 1.1. Components, Sources and Chemical Characteristics of Policosanol

Policosanol is a mixture of high-molecular-mass, very long chain fatty alcohols isolated from sugar cane wax (Saccharum officinarum, L) by hydrolytic cleavage and subsequent purification (Mas et al, 1999), or from other sources, such as rice wax, wheat germ, Grain Sorghum. Major components of policosanol are octacosanol (approximately 67%), triacontanol and hexacosanol with tetracosanol, heptacosanol, nonacosanol, dotriacontanol and tetratriacontanol existing as minor components (Mas et al, 1999; Castano et al, 2001). Policosanol composition varies depending on the source of material

and the method of preparation, and it is a poorly defined and highly variable mixture. Its complex constituents have not been fully characterized (Hargrove et al, 2004).

The chemical formula is $CH_3$-$(CH_2)$ n-$CH_2OH$ with chain length varying from 24 to 34 carbon atoms. For example, the formula of octacosanol is $CH_3$-$(CH_2)_{26}$-$CH_2OH$, triacontanol is $CH_3$-$(CH_2)_{28}$-$CH_2OH$.

## 1.2. Other Natural Potential Policosonal Sources

Free primary alcohols are found in many other natural waxes, including germ, kernel, seed coat, shell and shin (peel) of various nuts, seeds, fruits and cereals, the chrysalis of silkworm (Kawanishi et al, 1991), and beeswax (Tulloch and Hoffman, 1972). The presence of waxes in the diet and increased worldwide consumption of policosanol provide impetus to learn more about uptake, metabolism, mechanism of action of these very long chain fatty alcohols and acids.

True waxes are defined chemically as esters formed between long chain fatty acids and long chain alcohols. Waxes ester, plant waxes and beeswax contain a variety of very hydrophobic compounds (Table 1.1), including nonesterified very long chain hydrocarbons, alcohols, aldehydes, and acids. Plant waxes are found in intracellular oil droplets and also on the surface of plants. Unhydrolyzed beeswax produced by honeybees, contains about 23% hydrocarbons, 45% wax monoesters, 6% diesters of long chain alcohols with palmitic acid, 1% free alcohols, and 12% free fatty acids (Table 1). The main sources of commercial very long chain fatty alcohols are saponified beeswax and sugarcane wax (Hargrove et al, 2004). Because waxes are relatively abundant in cereal grains, bran, and germ, as well as in leaves, seeds, nuts, and unrefined oils (Kolattukudy 1976), the human diet has always contained modest levels of waxes.

Wax ester are hydrolyzed by a bile salt-dependent pancreatic carboxyl esterase, releasing long chain alcohols and fatty acids that are absorbed in the gastrointestinal tract (Hargrove et al., 2004). It is not clear whether natural waxes in which esters predominate are equally effective at lowering cholesterol. Wax hydrolysis may be partly due to pancreatic lipase, but a variety of carboxyl esterases could potentially cleave wax esters (Aneiros et al., 1995).

**Table 1.1** Major Wax Components of Typical Human Foods

| Source | % dry weight | Wax esters | Primary alcohols | Free acids | Aldehydes | Alkanes |
|---|---|---|---|---|---|---|
| **Honeycomb** | 93 | 35-45 | — | 8-12 | — | 14-23 |
| **Sugarcane** | — | 6 | 26 | 10 | 50 | 8 |
| **Wheat** | 0.4-0.7 | 9 | 17 | 3 | 36 | 9 |
| **Oats** | 0.38-0.9 | 7-21 | 5.6-14 | 8.8-14 | — | 8.8-15 |
| **Maize** | — | 14-62 | 14 | 14 | 9 | 17 |
| **Rice** | 0.1 | 35 | 40 | 0 | 10 | 15 |
| **Sorghum** | 0.2 | 4 | 34 | 24 | 32 | 1 |
| **Apple** | — | 18 | 6 | 20 | 2 | 20 |
| **Grape** | — | 9 | 40 | 7 | 12 | 1 |
| **Cabbage** | — | 4-22 | 2-9 | 2-9 | — | 36-40 |

Cited from Hargrove et al., 2004

## 1.3. Function of Policosanol

### 1.3.1. Cholesterol-lowering Effects

Independent confirmation of an effect of octacosanol on serum and liver cholesterol was provided when sugarcane rinds or wax extracted from sugarcane was found to lower cholesterol in rats (Sho et al., 1981; Sho et al., 1984). This effect was noted when wax was added to the diet at a level of 0.5g/100g, and purification of the active ingredient led to identification of long chain alcohols as cholesterol-lowering agents (Sho et al., 1983). The ability of policosanol to lower cholesterol or cholesterol synthesis has been demonstrated in the last decade in rats (Noa et al., 1995; Menendez et al., 1996), rabbits (Arruzazabala et al., 1994), dogs (Mesa et al., 1994), and monkeys (Rodriguez-Echenique et al., 1994), as well as in healthy volunteers (Hernandez et al., 1992), in patients with type II hypercholesterolemia (Pons et al., 1994; Canetti et al., 1995; Castano et al, 2000), in patient with type II diabetes (Torres et al, 1995; Crespo et al 1997) and in patients with high global coronary risk (Castano et al 1999).

Clinical studies have been carried out during short- and long-term feeding of policosanol in randomized placebo-controlled study designs (Table 1.2). In normocholesterolemic volunteers without dietary restrictions, 10 or 20 mg of policosanol per day given for 4 weeks decreased total and LDL cholesterol significantly and the

effects are dose dependent (Hernandez et al., 1992). Short-term studies over a 6- or 8-week period in patients with type II hypercholesterolemia indicated that 5 or 10 mg per day would lower total cholesterol by about 13% or 16% and LDL-C by about 18% or 22%, respectively (Pons et al., 1992; Aneiros et al., 1995). Two further short-term studies over 6-8 weeks used incremental dose of policosanol confirmed that the cholesterol-lowering effects are dose dependent and that, with 20 mg per day, LDL-C can be lowered by about 30% (Aneiros et al., 1993; Pons et al., 1994;).The ratio of total and LDL cholesterol to high-density lipoprotein cholesterol (HDL-C) can be substantially lowered in the order of 30%. HDL-C increased slightly but not significantly and triglyceride levels showed a variable response.

Long-term clinical studies performed over a 1-year period with use of 5mg per day of policosanol (Pons et al., 1994) or 10 mg per day of policosanol (Canetti et al., 1995) and over 2 years with use of 10 mg per day of policosanol (Canetti et al., 1995) show that the total and LDL cholesterol-lowering effects are maintained throughout this time period and that maximal effects are reached after 6 to 8 weeks of treatment. The increase in HDL-C seems to develop much more slowly than the decrease in apolipoprotein B-containing lipoproteins. Furthermore, the cholesterol-lowering effects do not differ substantially between the 5- and the 10-mg doses.

**Table 1.2 Randomized double-blind placebo-controlled trials of policosanol with the primary outcome parameter of Cholesterol lowering**

| Reference | Study population (n) | Policosanol dosage (mg/d) | Treatment duration (wk) | Baseline LDL-C (mmol/L)of treatment group | % change | | | | | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | LDL-C | TC | HDL-C | LDL/HDL | TC/HDL | TG | |
| Hernandez et al, 1992 | HV (38) | 2×5 | 4 | 2.9±0.7 | -10.0 (NS) | -10.7* | +2.6 (NS) | −22.4(NS) | −19.7* | −33.6* | 3-Group study design (2 dosages vs. placebo) |
| | | 2×10 | | 2.9±0.6 | -22.0* | -11.3* | +23.9* | −42.4* | −32.6* | −36.5(NS) | |
| Pons et al, 1992 | HC (56) | 1×5 | 8 | 5.1±0.7 | -17.7* | -13.1* | -3.3* | −14.1* | −9.1* | −13.7(NS) | |
| Aneiros et al, 1995 | HC (45) | 2×5 | 6 | 5.5±1.2 | -21.5* | -16.2* | +14.0 (NS) | −23.0* | −17.7* | −4.3(NS) | |
| Aneiros et al, 1993 | HC (33) | 2×5 | 6 | 5.0±0.8 | -21.2* | -16.7* | +2.9 (NS) | −24.9* | −21.0* | −23.3(NS) | Successive dose increases,6 wk each period (total 12 wk) |
| | | 2×10 | 6 | | -30.0* | -20.9* | +7.7(NS) | −33.8* | −26.0* | −12.1(NS) | |
| Pons et al, 1994 | HC (22) | 1×5 | 8 | 5.7±1.4 | -11.3* | -8 | +7.8(NS) | −15.3* | −12.5* | −2.7(NS) | Successive dose increases,8 wk each period (total 12 wk) |
| | | 2×5 | 8 | | -21.9* | -14.1* | +7.2(NS) | −25.6* | −18.4* | +7.8(NS) | |
| | | 2×10 | 8 | | -32.2* | -23.0* | +7.7(NS) | −34.6* | −27.2* | −10.5(NS) | |
| Pons et al, 1994 | HC (59) | 1×5 | 52 | 4.9±0.7 | -23.7* | -15.3* | +2.2(NS) | −25.3* | −17.0* | 0.0(NS) | |
| Canetti et al, 1995 | HC (97) | 2×5 | 52 | 4.9±0.7 | -27.5* | -16.3* | +25.9* | −37.1* | −28.0* | +17.5(NS) | |
| Canetti et al, 1995 | HC (69) | 2×5 | 104 | 5.4±1.0 | -24.8* | -18.3* | +11.2* | −32.6* | −26.6* | +13.0(NS) | HDL+21% after 52 wk,+14% after 78 wk, and+11.2% after 104 wk |

Only English language original publications are referenced. All studies are single-center and randomized placebo-controlled trials. TC, Total cholesterol, TG: Triglycerides; HDL-C, high-density lipoprotein cholesterol; HV, healthy volunteers; NS, not significant; HC, patients with hypercholesterolemia type II. *P< .05 between values at baseline and end of treatment. Cited from Couni-berthold et al: Policosanol: Clinical pharmacology and therapeutic significance of a new lipid-lowering agent. American Heart Journal 2002; 143: 356-365.

**Table 1.3 Randomized double-blind placebo-controlled trials with the primary outcome parameter of Cholesterol lowering in special patient populations**

| Reference | Study population (n) | Policosanol dosage (mg/d) | Treatment duration (wk) | Baseline LDL-C (mmol/L) of treatment group | % change | | | | | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | LDL-C | TC | HDL-C | LDL/ HDL | TC/ HDL | TG | |
| Torres et al, 1995 | HC and NIDDM (2 9) | 2×5 | 12 | 5.5±1.2 | −21.7* | −16.9* | +6.5 (NS) | -26.5* | -22.0* | -6.6 (NS) | See table 1.4 for another study in NIDDM patients 8 |
| Crespo et al, 1997 | HC and NIDDM (1 9) | 2×5 | 12 | 5.3±1.1 | −44.4* | −28.9* | +23.5* | -51.5* | -38.3* | -2.4 (NS) | |
| Castano et al, 1996 | HC and hypertension (5 8) | 2×5 | 52 | 5.2±0.8 | −19.1* | −13.0* | +17.1* | -24.2* | -20.0* | -8.0 (NS) | |
| Castano et al, 1995 | HC elderly patients (6 2) | 2×5 | 52 | 5.4±1.0 | −23.1* | −15.6* | +6.3 (NS) | -25.2* | -19.0* | -3.5 (NS) | Patients aged 60-80 y |
| Castano et al, 2000 | HC postmenopausal women (244 ) | 1×5 | 12 | 5.0±1.1 | −17.7* | −12.6* | +16.5* | -17.0 * | -16.7* | -4.6 (NS) | Successive dose increases, 12wk each period (total 24 wk) |
| | | 1×10 | 12 | | −25.2* | −16.7* | +29.3* | -29.3* | -27.2* | -1.5 (NS) | |
| Mas et al, 1999 | HC and > 2 risk factors (437) | 1×5 | 12 | 5.1±0.7 | −18.2* | −13.0* | +15.5* | -19.1* | -17.3* | -5.2* | Successive dose increases, 12 wk each period (total 24 wk) |
| | | 2×5 | 12 | | −25.6* | −17.4* | +28.4* | -32.5* | -28.8* | -11.9 (NS) | Another study of patients with multiple CHD risk factors. |
| Zardoya et al, 1996 | HC and hepatic dysfunction(4 6) | 1×5 | 12 | 5.2±0.5 | −19.1* | −13.6* | +11.5* | -25.5* | -21.1* | -10.2 (NS) | 4-Group study design (2 dosages vs. 2 placebo groups); high dropout rate (10/46) |
| | | 2×5 | | 5.4±1.2 | −22.3* | −15.4* | +17.9* | -32.2* | -26.1* | -0.0(NS) | |

Only English language original publications are referenced. All studies except for the third and sixth are single-center trials. TC, Total cholesterol; TG, Triglycerides; HC, patients with hypercholesterolemia type II. CHD, coronary heart disease; NIDDM, non-insulin-dependent diabetes mellitus; NS, not significant. *P< .05 between values at baseline and end of treatment. †P=.06. Cited from Couni-berthold et al: Policosanol: Clinical pharmacology and therapeutic significance of a new lipid-lowering agent. American Heart Journal 2002; 143: 356-365.

Studies were also conducted in special patient populations (Table 1.3). Because Coronary heart disease (CHD) is the main cause of death in patients with diabetes mellitus, with its characteristic dislpidemia being a major risk factor, the efficacy of new lipid-lowering agents must be comprehensively investigated in this patient group. A double-blind placebo-controlled study using 10 mg of policosanol per day in patients with controlled non-insulin-dependent diabetes mellitus (NIDDM) and hypercholesterolemia show significant reductions in total cholesterol of 16.9%, in LDL-C of 21.7%, and a nonsignificant increase in HDL-C of 6.5% (Torres et al., 1995). Triglyceride concentrations, glucose levels, and hemoglobin (Hb) $A_1$ values remained unchanged. In another study in diabetic patients, LDL-C levels decreased by 44% and HDL-C increased by 23.5% (Crespo et al., 1997). The ratios of total and LDL-C to HDL-C were significantly improved. It remains unclear why the effects on cholesterol were so large in this latter study, but because of its pronounced effect on the ratio of LDL-C to HDL-C policosanol seems to be very promising for the treatment of diabetic dyslipidemia.

In a study in patients with type II hypercholesterolemia and concomitant hypertension, it was shown that policosanol significantly lowered systolic blood pressure by a mean of 10 mm Hg (Castano et al., 1996). Beneficial effects on blood pressure have not been confirmed in other studies.

An open-label study over 1 year using 20 mg per day in patients with a high CHD risk (88% family history of premature CHD, 71% hypertension, 60% previous coronary events, 60% severe hypercholesterolemia >7.8 mmol/L) demonstrated an impressive decrease in LDL-C of 44.8% and an increase in HDL-C of 68.5% (Castanol et al., 1999).

The majority of these studies have been conducted in Cuba. Then it is important to assess the potential functions of policosanol in different regions and background diets with different animal models and ethnic populations.

### 1.3.2. Comparison Between Policosanol and other Lipid-lowering Drugs

Of special interest in judging the efficacy of policosanol are trials in which the effects of established lipid-lowering medications, such as statins or fibrates, are compared with those of policosanol (Table 1.4). Comparison of simvastatin (10 mg per day) with policosanol (10 mg per day) in elderly patients with moderate primary

hypercholesterolemia showed that both drugs have at these dosages similar efficacy in lowering total cholesterol and LDL-C (Ortensi et al., 1997). In another short-term study (6 weeks) comparing the efficacy of policosanol (10 mg per day) versus pravastatin (10 mg per day), the reduction in total cholesterol was similar with both drugs, whereas the decrease in LDL-C and the increase in HDL-C were more pronounced during policosanol treatment (Benitez et al., 1997). This finding could be confirmed in a recent study in elderly patients treated for 8 weeks (Castano et al., 1999). In patients with non-insulin-dependent diabetes mellitus (NIDDM) comparing policosanol (10 mg per day) with lovastatin (20 mg per day) also showed that policosanol was slightly superior in lowering total and LDL cholesterol and increasing HDL-C (Crespo et al., 1999). This finding was confirmed in a study using the same dosages in patients with multiple CHD risk factors (Castano et al., 2000).

### 1.3.3. Other potential functions of policosanol

In addition to cholesterol-lowering, there is evidence from various animal studies that policosanol prevents onset of spontaneously and experimentally induced atherosclerotic lesions. Policosanol seems to have positive pleiotropic effects on smooth muscle cell proliferation, LDL oxidation, and platelet aggregation. Studies have shown that policosanol reduces lipofundin-induced atherosclerotic lesions in rabbits and rats, including foam cell formation (Noa and Mas, 2005; Noa, et al., 1995) and prevents the development of foam cells in carrageenan-induced granulomas in rats (Noa et al., 1996). Policosanol also inhibits smooth muscle cell proliferation induced in rabbit cuffed artery (Noa et al., 1998; Noa et al., 2001) and in arterial wall damage induced with forceps on the central artery ear of the rabbit (Noa et al., 1998). Furthermore, policosanol administered long term up to 54 weeks lowered serum cholesterol and prevented development of atherosclerotic lesions in Macaca arctoides monkeys (Rodriguez et al., 1994). Policosanol decreased susceptibility of lipoproteins to peroxidation in rats (Fraga, et al., 1997) and in humans in vivo (Menendez, et al., 2000). A recent animal study in monkeys (Noa and Mas R, 2005) showed that policosanol reduced the presence of macrophages and the occurrence of apoB, whereas increased apoA-1 localization was found in aortic atherosclerotic lesions compared with control monkeys. The inhibitio

**Table 1.4** Randomized double-blind placebo-controlled trials of policosanol with the primary outcome parameter of Cholesterol lowering

| Reference | Study population (n) | treatment and dosage (mg/d) | Treatment duration (wk) | Baseline LDL-C (mmol/L) of treatment group | % change | | | | | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | LDL-C | TC | HDL-C | LDL/ HDL | TC/HDL | TG | |
| Ortensi et al, 1993 | HC elderly patients (53) | Policosand2×5 | 8 | 5.0±0.8 | −17.9* | −14.7* | −1.7(NS) | −15.4* | −12.4* | −13.8* | Patient age 60-77 y (Baseline triglyceride concentrations were significantly higher in policosanol group.) HC type 11b |
| | | Simvaslafin2×5 | | 5.1±0.5 | −19.8* | −15.2* | −2.9(NS) | −16.6* | −11.8* | −8.7* | |
| Benitez et al, 1994 | HC (24) | Policosand2×5 | 6 | 5.0±0.5 | −24.2* | −15.7* | +13.6* | −33.0* | −25.7* | −8.7* | |
| | | Policosand1×10 | | 5.0±0.2 | −19.6* | −15.3* | +4.7(NS) | −22.8* | −18.7* | −13.7* | |
| Castano et al, 1999 | HC elderly patients with | Policosand1×10 | 8 | 4.6±0.6 | −19.3* | −13.9* | +18.4* | −28.3* | −24.4* | −14.1* | Patients' age 60-80 y (Additional outcome parameters were effects on platelet aggregation) |
| | Multiple CHD risk factors (68) | Policosand1×10 | | 4.6±0.6 | −15.6* | −11.8* | +5.6* | −18.9* | −15.7* | −3.4(NS) | |
| Crespo et al, 1999 | NIDDM (53) | Policosand1×10 | 12 | 5.3±1.0 | −20.4* | −14.2* | +7.5* | −23.7* | | −18.4(NS) | |
| | | Lovestatin1×20 | | 5.3±1.0 | −16.8* | −14.0* | −2.8(NS) | −14.9* | | −0.5(NS) | |
| Castano et al, 2000 | HC and > 2 CHD risk factored (59) | Policosand1×10 | 12 | 4.7±0.9 | −32.4* | −22.4* | +14.3* | −39.3* | −32.0* | −22.5* | |
| | | Lovestatin1×20 | | 4.6 ±0.8 | −27.6* | −19.8* | +3.7(NS) | −32.8* | −25.2* | −24.0* | |
| Marcello et al, 2000 | CD(29) | Bezafibrate1×400 | 8 | 4.8±0.8 | −10.7* | −9.2* | +8.0* | −13.3* | −13.8* | −29.2* | Baseline triglyceride levels: 3.3±0.9 3.1±0.7 |
| | | Bezafibrate1×400 Plus policosanol 1×10 | | 5.3±1.0 | −27.7* | −20.8* | +14.5* | −36.8* | −29.8* | −30.2* | |

Only English language original publications are referenced. All studies are single-center trials. TC, Total cholesterol; TG: triglycerides; HC, patients with hypercholesterolemia type II. NIDDM, non-insulin-dependent diabetes mellitus; NS, not significant. CD, combined dyslipidemia. *P< .05 between values at baseline and end of treatment. Cited from Couni-berthold et al: Policosanol: Clinical pharmacology and therapeutic significance of a new lipid-lowering agent. American Heart Journal 2002; 43: 356-365.

effect by policosanol on macrophages content in aortas of Macaca arctoides monkeys is 62.5%, 74.7% at dose of policosanol 2.5 and 25 mg/kg body weight, respectively.

Neointimal formation due to smooth muscle cell proliferation is crucial in the progression of the atherosclerotic plaque. Accelerated proliferation of smooth muscle cell appears to be a cause of early coronary occlusion in patients undergoing heart transplantation, coronary artery bypass graft and percutaneous transluminal coronary angioplasty. Thus, factors controlling smooth muscle cell proliferation are important development for atherosclerosis (Ip et al., 1990; Cooper et al., 1991). In rabbits and rats policosanol can reduce the development of meointimal formation (smooth muscle cell proliferation) (Noa et al., 1998) as well as atherosclerotic lesions, including foam cell formation (Noa et al., 1995; Noa et al., 1996).

Policasonal has pronounced antiplatelet aggregation effects in animal models and there is evidence that a decrease in thromboxane $B_2$ and an increase in prostacyclin levels are involved (Arruzazabala et al., 1992; Arruzazabala et al., 1993; Carbajal et al., 1994). Antiplatelet effects were also shown in healthy volunteers during single or continuous dosing with policosanol (Arruzazabala et al., 1996; Valdes et al., 1996) and in patients with hypercholesterolemia (Castano et al., 1999; Arruzazabala et al., 1998). Single doses (10–50 mg) of policosanol show that it inhibits adrenaline- and ADP-induced aggregation in a moderate dose-dependent fashion, but leave collagen-induced aggregation unchanged (Valdés et al., 1996). Several short term previous studies, all randomized, double-blind and placebo-controlled, have investigated the antiplatelet effects of repeated doses of 10 mg/day policosanol administered for 1–4 weeks to healthy subjects and for 8 weeks to patients with type II hypercholesterolaemia. The results showed significant reductions in the aggregation induced by arachidonic acid (AA), collagen and ADP up to 69.5, 41.1 and 24.1%, respectively (Arruzazabala et al., 1996; Scazziota et al., 1996; Carbajal et al., 1998; Arruzazabala et al, 1998). Because a relationship between serum cholesterol and thromboxane formation and platelet hyperreactivity may be associated with hypercholesterolemia, the antiplatelet properties of policosanol seem to be the most promising additional feature of this new lipid-lowering drug.

Studies also show that policosanol can improve exercise performance of coronary heart disease patients (Stusser et al., 1998) and increase muscle endurance (Kabir et al.,

1995). In ovariectomized rats, investigators (Noa, et al., 2004) demonstrated that policosanol prevented bone loss and decreased bone resorption, by preventing the increase in the number of osteoclasts and their surface area induced by ovariectomy. The results suggest that policosanol should be potentially useful in preventing bone loss in postmenopausal women.

However, all these potential beneficial effects have not been confirmed by other independent studies. Thus more studies are warranted before a conclusion can be drawn.

## 1.4. Safety of Policosanol

Policosanol is only effective when used on a repeated basis, this toxicity related to chronic use is of primary concern. Studies examining the safety of poicosanol are performed in three key areas: acute and chronic toxicity, carcinogenicity, and mutagenicity. In 1994, Aleman et al. (Aleman et al., 1994) demonstrated that policosanol administered to rats at 50 to 500 mg/kg/day for 12 months revealed no treatment-related toxicity with regards to either clinical observations or blood biochemistry. In vitro and in vivo mutagenicity studies showed that policosanol was not related to any genotoxic effects on somatic or germinal cells (Rendon et al., 1992). A carcinogenicity study conducted in mice revealed no drug-related increase in the occurrence of malignant or benign neoplasm when policosanol was administered at 50 to 500 mg/kg/day for 18 months (Aleman et al., 1995). When used in long-term clinical trials, policosanol has been shown to be well tolerated and safe (Pons et al., 1994; Castano et al., 2002; Mas et al., 1999). In a recent study examining the effect of policosanol administration at 5 to 10 mg/day for 12 months in older patients with type II hypercholesterolemia and hypertension, total adverse events were less frequent in the policosanol-treated group (9.8%) than in the group receiving placebo (17.7%). On the rare occasion that adverse events were reported, these events included slight weight loss, polyuria, and headache (Mas et al., 1999). Thus, studies performed to date demonstrate a wide safety margin for policosanol when administered long term. Future research should focus on defining a more precise tolerability profile for humans at doses greater than 10 mg/day and for periods exceeding 12 months.

## 1.5. Potential Underlying Metabolism and Mechanism of Policosanol

### 1.5.1. Metabolism of Short Chain Alcohol

The metabolism of fatty alcohol (in Fig. 1.1) seems to occur as a cycle. The concept of a fatty alcohol cycle derives from the finding that fatty alcohol within the intact cell is simultaneously synthesized from fatty acid and oxidized back to fatty acid. The enzymes catalyzing these reactions are distinct. Support for this fatty alcohol cycle arises from the existence of human inborn errors of metabolism associated with blocks in specific reactions (Table 1.5). The fatty alcohol cycle is not closed to entry of intermediate metabolites, such as dietary lipids or catabolic products of other lipids. Fatty alcohol is utilized as a substrate for the synthesis of wax esters and ether lipids (Rizzo, 1998).

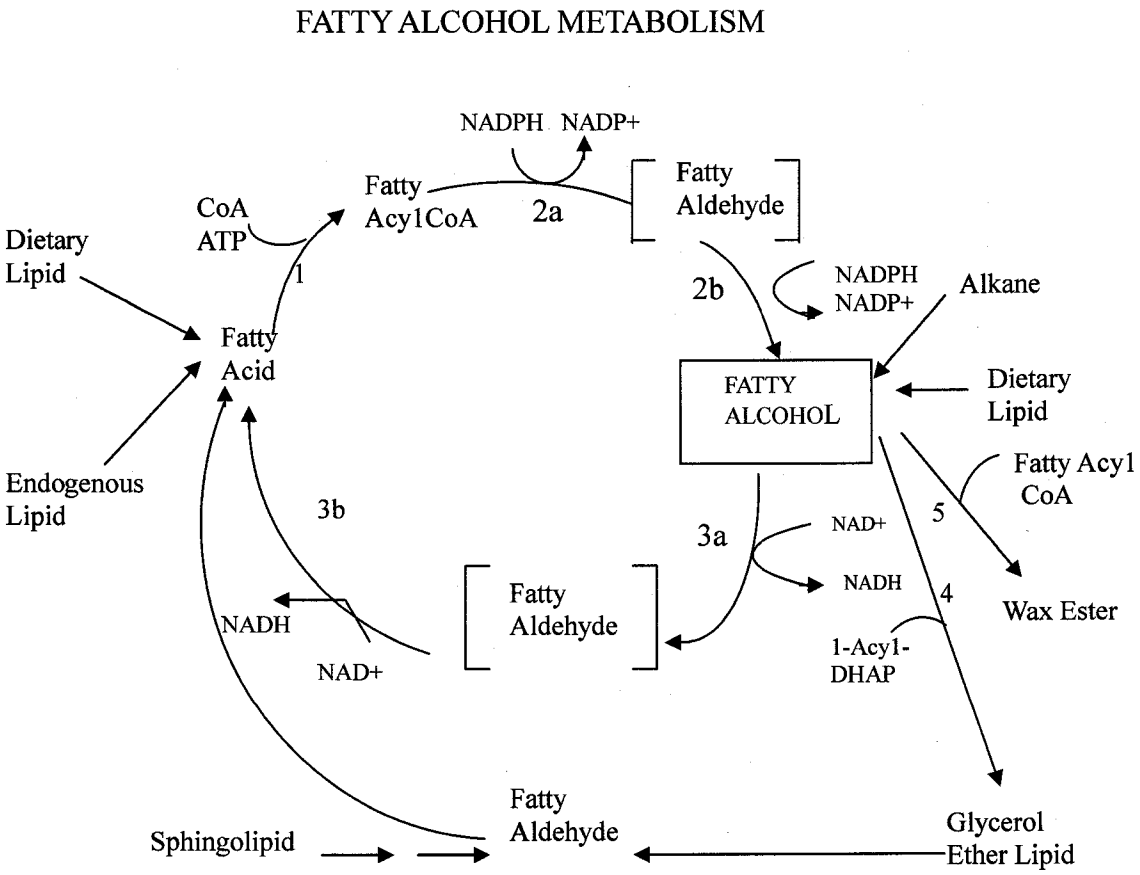


**Figure 1.1 Fatty alcohol metabolism**

Numbers refer to reactions catalyzed by the following enzymes: 1, acyl-CoA synthetase; 2, acyl-CoA reductase; 3, fatty alcohol: $NAD^+$ oxidoreductase complex; 3A, fatty alcohol dehydrogenase; 3B, fatty aldehyde dehydrogenase; 4, alkyl-DHAP synthase; 5, acyl-CoA:fatty alcohol acyl transferase. Figure is citied from William B. Rizzo (Rizzo WB, 1998).

The existence of inborn errors of fatty alcohol metabolism was first revealed by the recognition that patients with inherited disorders of peroxisomal biogenesis had impaired lipid synthesis (Heymans et al., 1983; Heymans et al., 1984; Schutgens et al., 1984; Datta et al., 1984). These patients were initially diagnosed by their overlapping clinical features and the shared biochemical abnormalities associated with global peroxisomal dysfunction. Subsequently, two isolated inborn errors of fatty alcohol metabolism have been identified, one of which is not a peroxisomal disorder. Significantly, all of the disorders are associated with severe neurological disease. These inherited diseases include isolated defects in the oxidation of fatty alcohol to fatty acid (Sjo¨gren-Larsson syndrome) and deficient incorporation of fatty alcohol into ether lipids (isolated alkyl dihydroxyacetone phosphate synthase deficiency). In addition, disorders of peroxisomal biogenesis (Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease) and peroxisomal protein import (rhizomelic chondrodysplasia punctata) have impaired ether lipid synthesis along with other associated defects in peroxisomal metabolism (Table 1.5).

### 1.5.2. Potential Underlying Metabolism of Policosanol

Evidence suggests that fatty alcohols, aldehydes, and acids are interconverted (Fig. 1.1) (Rizzo et al., 1987; Rizzo 1998). However, it is unclear if very long chain fatty alcohols have the same metabolism cycles.

Animal and cell culture studies to define the mechanism of policosanol action have also been conducted. In vivo studies in rats and rabbits have shown that policosanol decreases hepatic cholesterol synthesis and, presumably as a result, increases hepatic expression of LDL receptors (Menendez et al., 1996; Menendez et al., 1997). The block evidently occurs at a point proximal to mevalonic acid (the product of HMG-CoA reductase), since policosanol does not impair the ability of liver to convert exogenous

**Table 1.5 Inherited Disorders Associated with Abnormal Fatty Alcohol Metabolism**

| Disorder | Genetic defect | Biochemical defect | Elevated plasma fatty alcohol | Clinical symptoms |
|---|---|---|---|---|
| Sjogren-Larsson syndrome | FALDH | FAIDH and FAO deficiency | Yes | Mental retardation, spasticity, ichthyosis |
| Rhizomelic.chondrodysplasia punctata | PEX7 | Deficient peroxisomal import of PTS-2 proteins→defective ether lipid synthesis. Impaired phytanic acid oxidation abnormal processing of peroxisornal thiolase | Yes | Psychomotor retardation chondrodysplasia punctata with rhizomelic limb shortening hepatomegaly dysmorphic features cataracts ichthyosis |
| Isolated alkyl-DHAP synthase deficiency | ? | Alkyl-DHAP synthase deficiency→defective ether lipid synthesis | ? | RCDP-like |
| Peroxisomal biogenesis disorders (Zellweger syndrome neonatal ALD. infantile Refsum disease) | PEX1, PEX2, PEX5, PEX6, PEX12 | Defective peroxisome biogenesis or protein import →deficient ether lipid synthesis. Impaired phytanic acid and very long-chain fatty acid oxidation, pipecolic acid accumulation, defective bile acid synthesis | ± | Psychomotor retardation dysmorphic facies hepatonegaly failure-to-thrine, hypotonia seizures |

FALDH= Fatty aldehyde dehydrogenase; FAO= fatty alcohol:NAD1 oxidoreductase; HDAP= alkyl dihydroxyacetone phosphate; RCDP= Rhizomelic Chondrodysplasia Punctata;
Cited from: Rizzo WB. Inherited disorders of fatty alcohol metabolism. Mol Genet Metab 1998; 65:63-73.

mevalonic acid to cholesterol. A recent report examining the impact of policosanol on cultured fibroblasts further clarifies this issue (Menendez et al., 2001). Policosanol has been found to decrease the expression of HMG-CoA reductase in fibroblasts in a dose-dependent manner; however, unlike statins, policosanol does not directly inhibit this enzyme. Of particular note is the fact that policosanol failed to decrease HMG-CoA expression by more than about 50%, even at the highest concentration tested; this provides a satisfying rationale for the safety of policosanol in animal toxicology and in clinical experience. The impact of policosanol on cholesterol metabolism thus appears to be analogous to that of tocotrienols and certain other natural isoprenoids which can inhibit HMG-CoA reductase expression by a feedback mechanism (Parker et al., 1993; Elson et al., 1999). The above rationale was further supported by another independent cell culture study (Singh et al., 2006), showing that HMG-CoA reductase activity is down-regulated by policosanol and suggesting that policosanol or a metabolite decreases HMG-CoA reductase activity by activating AMP-kinase. An animal study showed that in addition to the effect on HMG-CoA reductase, the cholesterol-lowering activity of policosanol is partially mediated by inhibition of bile acid absorption (Ng et al., 2005).

A recent study showed that oral intake of D-003, a mixture of very long chain fatty acids (VLCFA), isolated from sugar cane wax, prevents casein-induced endogenous hypercholesterolemia in rabbits(Menéndez et al., 2004). Present data do not indicate whether the cholesterol-lowering effect of policosanol requires conversion of fatty alcohols to fatty acids. It also not known whether pure VLCFA can lower cholesterol levels more efficiently than policosanol or a mixture of policosanol with VLCFA.

## 1.6. Negative results from other research groups

### 1.6.1. Negative results of policosanol as lipid-lowering agents

Most of the above studies have been done in Cuba and in the same research institute in the last decade. These results show that policosanol is a promising new agent, which is safe, effective and cost- efficient. Policosanol has raised the close attention of many other researchers in USA., Canada, Australia, Germany, China, South Africa. Independent studies done by these scientists do not fully support previous reports from Cuban studies. Some authors report totally conflicting results in similar animal models as

well in clinical trials.

For example, an animal study was conducted recently to examine the effect of policosanol supplementation in the diet on the fecal excretion of neutral and acidic sterols in hamsters (Ng et al., 2005). The results show that the cholesterol-lowering activity of policosanol is partially mediated by inhibition of bile acids absorption, which is another potential mechanism instead of the effect of HMG-CoA reductase. Excretion of cholic, chenodeoxycholic, lithocholic, and ursodeoxcholic acids was not dose-dependent on policosanol level in the diet at levels of 0.38-1.5 g/kg (equivalent to 4-16mg/hamster/day), although the excretion of fecal policosanol was proportional to the amount of policosanol in the diet.

The same animal model was used in another study to examine the effects of policosanol and phytosterol, alone and in combination, on lipid profiles, cholesterol biosynthesis, and tissue histopathological changes in hamsters (Wang et al., 2003). Results are sharply conflicting, and did not show that policosanol intake at 25 mg/kg body weight (BW) from sugar cane or ricewax had any significant effect on lipid profiles. It is not clear if the discrepancies between the two independent studies with same animal model were caused by animal diet composition or other factors. Another animal study was carried out on rabbits (Murphy et al., 2004) with policosanol extracted from sugar cane and a similar product derived from winteriser cake containing policosanol during sunflower oil production (SFP). Again, the results do not support the notion that policosanol extracted from either sunflower oil cake or sugar cane can lower cholesterol in this animal model.

Clinical studies have been also conducted in randomised, placebo-controlled, double-blind study designs. In a 4 wk short-time clinical study feeding of wheat germ policosanol (WGP), which is similar to the composition of sugar cane policosanol, showed that WGP at 20 mg/d had no beneficial effects on blood lipid profiles in subjects with normal to mildly elevated cholesterol concentrations (Lin et al., 2004). At 20 mg/d policosanol for 12 weeks, another longer clinical study also found no significant effect on serum lipid levels in hypercholesterolaemic and heterozygous familial hypercholesterolaemic patients when compared with placebo intake (Greyling et al., 2006).

Another clinical trial was carried out among patients with hypercholesterolemia or combined hyperlipidemia for a 12-week treatmen at usual and high doses of 10, 20, 40, or 80 mg/d of policosanol (Berthold et al., 2006). Dosages tested did not demonstrate any significant reduction in total cholesterol, high-density lipoprotein cholesterol (HDL-C), very low-density lipoprotein cholesterol, triglycerides, lipoprotein(a), and ratio of total or LDL-C to HDL-C.

Most of the recent animal studies as well as clinical trials conducted outside of Cuba do not support the previous effect of policosanol as a cholesterol-lowering agent.

### 1.6.2. Negative results of policosanol as antioxidants

Previous results indicate that low-density lipoprotein (LDL) oxidation is a key factor in initiation and progression of atherogenesis (Steinberg et al., 1989; Aviram, 1993). Once oxidized, LDL is actively taken up by macrophages, leading to the formation of lipid-laden foam cells, the hallmark of early atherogenic lesions (Brwonn et al., 1983). A variety of studies in Cuba have shown that policosanol inhibits lipid peroxidation in experimental models, as well as in humans. When policosanol was administered orally (100 and 250 mg/kg) for up to 4 weeks, a partial prevention of rat in vitro microsomal lipid peroxidation .was observed (Fraga et al., 1997). The formation of TBARS (thiobarbituric acid reactive substances) in microsomes isolated from policosanol treated rats was significantly decreased by about 50%. The investigators also found oral administration of policosanol in rats provides a partial inhibition of lipid peroxidation. Further study in vitro demonstrated that policosanol, in addition to its cholesterol-lowering effect, has other properties that enable it to reduce the potential of lipoprotein to undergo lipid peroxidation (Menendez et al., 1999). Similar findings have been shown in healthy human volunteers (Menendez et al., 2000; Menendez et al., 2001). These results suggest that policosanol has a protective effect on lipid peroxidation and thus inhibits development of atherosclerosis.

Antioxidant activity of policosanol is not supported in a recent human low-density lipoprotein (LDL) study (Ng et al., 2005). The results demonstrate that LDL was rapidly oxidized in LDL with the addition of 60μM policosanol compared with a-tocopherol, while the induction time of latter was at least 8 h. The investigators observed that

policosanol had no or weak scavenging activity on DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals as compared with ą-tocopherol. The policosanol tested scavenged only 3% of DPPH free radicals while ą-tocopherol scavenged 94% of DPPA free radicals. These results demonstrated that policosanol had no anti-LDL oxidation, and do not support the rationale that policosanol can offer any protection against free radical-associated diseases.

It is unknown whether the discrepancies between recent animal and human studies result from difference in species, dosage, composition, treatment term and delivery form of the test materials. More studies are warranted to investigate beneficial effects of policosanol in independent experiments. The metabolism and mechanism of policosanol action and the activity of individual very long chain alcohols remains to be elucidated.

## 1.7. Caco-2 cells and Monolayer Model

Caco-2 cells were isolated from a 72 year-old Caucasian male presenting with an adenocarcinoma of the colon (Fogh et al, 1977). Cells from this patient, once isolated, proliferated rapidly in culture and were able to induce further tumors when injected into nude mice. Interestingly, in a subsequent study of 20 human colon tumor cell lines, Caco-2 alone showed the ability to undergo spontaneous differentiation to develop a number of characteristics more commonly associated with small intestinal enterocytes (Chantret et al, 1988). This was the first indication that Caco-2 cells might be a useful tool to model events occurring in small intestinal enterocytes. These cells spontaneously differentiate to an enterocyte-like phenotype when cell monolayers reach confluency and are maintained using conventional culture conditions (Hildalgo et al. 1989; Pinto et al. 1983). During the early phases of differentiation, the cells express both colonocyte- and enterocyte-specific proteins (Engle et al. 1998). As differentiation proceeds, colonocyte-specific gene expression decreases and morphological and biochemical characteristics of enterocytes develop (Failla and Chitchumroonchokchai, 2005). The cells require a minimum of 10 - 12 days after the monolayer reaches confluency to mature to the enterocyte-like state (Hildago et al. 1989) and 21 - 25 days for effective synthesis and secretion of lipoproteins (Mehran et al. 1997).

To better reproduce the steric conditions existing in the intestine in vivo, Caco-2

cells were cultured on permeable filter supports that allow free access to nutrients or dugs to the two sides of the cell monolayer. Since these conditions led to improved morphological and functional differentiation, they have been proposed, and since then extensively utilized, as a more physiological model for intestinal transport and toxicity studies (Artursson, 1990; Hidalgo et al, 1989; Sambuy et al, 2005).

Trypan blue exclusion assay and MTT assay are reported techniques to assess or evaluate cytotoxicity and cell viability. Transepithelial electrical resistance (TEER) after confluence and permeability of marker molecules have been used to monitor the integrity of the cell layer and ultrastructural morphology. Transmission electron microscopy, has been utilized to check for morphological differentiation.

It has been reported that Caco-2 cells grown in these permeable supports in bicameral chambers, under control conditions for more than 20 days, spontaneously differentiate into an ileal-like polarized cells monolayer which develops brush border microvilli, tight junctions, and both specific small intestinal enzymes and gene products (Hidalgo et al, 1989; Pinto et al, 1983; Matsumoto et al, 1990). For this reason, this cell line has been widely used as an in vitro model system in many studies concerning the evaluation of intestinal absorption of substances present in food, contaminants or drugs. These studies include absorption of amino acids (Costa et al. 2000; Nicklin et al. 1995), cholesterol (Nagaoka et al. 2002), fatty acids (Ranheim et al. 1994), iron (Glahn et al. 2002; Au and Reddy 2000), vitamins B6 (Mackey et al. 2004), and bioactive polyphenols (Steensma et al. 2004; Vaidyanathan and Walle 2003; Walgren et al. 1998), saponins (Hu et al. 2004), and many others including drugs.

Thus, the utility of differentiated cultures of Caco-2 cells as a model for investigating the characteristics and regulation of the transport and metabolism of dietary compounds by absorptive epithelial cells is widely accepted.

## 1. 8. Limitations of the Caco-2 cell monolayer model

It is also important to note that some characteristics of differentiated Caco-2 cell monolayer differ from those of small intestinal enterocytes. First and most obvious, the cells originate from a human colonic carcinoma rather than normal small intestine (Pinto et al. 1983). Second, the cell line is genetically and phenotypically heterogeneous, cell

culture studies do not provide information on individual responses to nutrients. Third, the transepithelial resistance associated with assembly of tight junctions in Caco-2 cells is more characteristic of colonic epithelium than small intestinal epithelium (Delie and Rubas 1997). Finally, Caco-2 cells use the glycerol 3-phosphate pathway for the synthesis of triacylglycerols, whereas the small intestinal epithelium uses the monoacylglycerol pathway (Levy et al., 1995). Some of the differences indicated are offset by standardization of procedures associated with the seeding, growth and maintenance of Caco-2 cells and the design of studies using this cell line. Factors that must be strictly controlled to minimize genetic and phenotypic "drift" and to facilitate comparison of results within and among laboratories include source of cells, range of passage numbers used for investigations, composition of incubation medium, pH used for uptake studies, degree of maturation of cells at time of experimentation, and composition and porosity of support material for cells for transport studies (Bailey et al. 1996).

# Chapter 2

# RESEACH PLAN

## 2.1. Rationale

The cholesterol-lowering effect of policosanol is controversial. Many studies have been performed in the last decade in animal models and through clinical trials. There is evidence that policosanol inhibits platelet aggregation and lipid peroxidation, decreases endothelial damage and smooth muscle cell proliferation (Arruzazabala et al., 2001; Arruzazabala et al., 2002; McCarty, 2002), improves exercise performance of coronary heart disease patients (Stusser et al., 1998), and increases muscle endurance (Kabir et al., 1995). The metabolism and mechanism of policosanol effects remain poorly understood. Further studies about potential functions, metabolism and mechanism of policosanol action are warranted.

Rizzo et al. suggests that very long chain fatty alcohols, aldehydes, and fatty acids are interconverted (Rizzo, et al., 1987&1988). It seems that long chain fatty alcohols will modify the fatty acids. It is unclear if very long chain fatty alcohols will be degraded to shorter chain fatty acids or alcohols, and it is also not known if each long chain fatty alcohol has the same metabolic characteristic.

Caco-2 cells, derived from a human with colon adenocarcinoma, have been widely used as an in vitro model system in many studies concerning the evaluation of intestinal uptake, metabolism and transport of nutrients, drugs and contaminants. These cells retain the ability for spontaneous differentiation that mimics normal intestinal ontogeny and epithelial crypt-to –villus migration (Pinto et al., 1983; Briske-Anderson et al., 1997).

Very few studies of policosanol action have been conducted in cell culture. No study has been done in Caco-2 cells. It is therefore of interest to examine the effect of policosanol on Caco-2 proliferation, the absorption rate of policosanol by these enterocyte-like intestine-similar cells, and the effects of policosanol on cell lipid profile after absorption.

## 2.2. Hypothesis

1. Policosanol can be absorbed by Caco-2 cells and will be oxidized and degraded into short-chain fatty acids.
2. Policosanol will modulate the lipid profile after being absorbed.

## 2.3. Research Objectives

The objectives of this thesis research are to examine:

1. The effect of policosanol on Caco-2 cells proliferation.
2. The absorption rate of policosanol by Caco-2 cells
3. Determine if policosanol/octacosanol is converted to shorter chain fatty acids or fatty alcohols after being absorbed.
4. Determine how policosanol affect the lipid profile and whether policosanol have a potential cholesterol-lowering effect or not.

# Chapter 3

# MATERIALS AND METHODS

## 3.1. Policosanol and individual standards

Policosanol was obtained from Mcgill University (Montreal, Quebec, Canada), it is sugar cane extract from Degussa bioactives (Champaign, IL, USA). Individual standards; tetracosanol, hexacosanol, heptacosanol, octacosanol and triacontanol were purchased from Sigma (St. Louis, MO, USA), 99.0% GC purity. The reagent: N-methyl-N-(trimethylsilyl) trifluoracetamide (MSTFA) was purchased from Sigma (St. Louis, MO, USA), 99.0% GC purity.

The composition of policosanol was determined using gas liquid chromatography (GC) with a minor modification of the Gonzalez-Canavaciolo and Magraner-Hernandez (1999) method. For the policosanol powder or dried aliquot of chloroform solution, 0.05 mL of N-methyl-N-(trimethylsilyl) trifluoroacetamide was added, and heated in heating module (Pierce chemical company, IL, USA) at 65℃ for 15 min to make trimethlsilyl (TMS) ether derivatives. The standard for other individual very long chain alcohols were prepared and derivatized in the same manner. Eicosanol (C20) (St. Louis, MO, USA) was used as the internal standard.

## 3.2. Capillary Gas Chromatography of policosanol

A Varian star 3400X gas chromatograph with a flame ionization detector (FID), and computerized data processor was used for policosanol analysis. The chromatographic separations were done using a fused-silica capillary column HT-5 (30m×0.25mm I.D., 0.5μm film thickness, SGE International Pty. Ltd). Conditions for gas chromatography were: injector temperature, 300 ℃; detector temperature, 300 ℃; oven temperature gradient, from 100 to 200℃ at 40℃/min, then increased by 10℃/min from 200 to 320℃ and subsequently kept at 320℃ for 30min. In the optimized system, the hydrogen and air flow-rates were 30 and 300 ml/min, respectively. Argon was used as a carrier gas at a flow-rate of 1 ml/ml. Injector parameters: split-splitless mode, septum purge flow-rate of 5 ml/min and a spilt-vent flow-rate of 45 ml/ml and closed for 60 s. A Varian Vista 654

Data System was used to analyze resolved peaks and to quantify long chain alcohols. Aliquots of 1-2μl were manually fast-injected with a 10μl Hamilton syringe into the injector. To quantify peaks, internal standard Eicosanol (C20) was used.

## 3.3. Preparation of policosanol/octacosanol micelle

For the cell viability assessment, phosphatidylcholine (PC) in chloroform (Sigma-Aldrich Canada Ltd., Oakville Ontario, Canada) was evaporated to dryness under a stream of nitrogen at 35℃. For the stock solution, phosphatidylcholine (PC) and policosanol were mixed together at 5:1 (w/w) ratio in Earle's Minimum Essential Medium supplemented with 4% fetal bovine serum and 1% antibiotic. This mixture was sonicated for 30 seconds and vortexed. The solution was filtered via sterile filter (5.00μm, 25mm diameter, Millipore, Carrigtwohill Ireland) to make a sterilized micelle. This solution was diluted to different concentrations with a micelle containing the same amount of PC, but without policosanol. BSA (bovine Serum Albumin) was also used as a carrier instead of PC when preparing policosanol or octacosanol micelles. The fixed concentration of BSA was 0.2% (w/w).

For study of policosanol metabolism, the same methods were followed, but the concentration of policosanol in the micelle was determined according to the cell viability result. Control cells were treated with micelle only containing the same amount of PC without policosanol.

## 3.4. Cells and cell culture

The human colon carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (HTB-37, Rockville, MD, USA). Cells were maintained and subcultured at 37℃ in an atmosphere of 5% $CO_2$, 95% air. When cells were 80% confluent, they were subcultured at a density of 1.6 $\times 10^4$cells/cm$^2$ on 25-cm$^2$ flasks using 0.25% trypsin-2.65 mM EDTA, and the medium was changed every 2 days. The complete medium for cell maintenance consisted of HEPES/carbonate-buffered EMEM (Earle's Minimum Essential Medium, GIBCO, Grand Island, NY) supplemented with 10% (v/v) Fetal Bovine Serum (GIBCO) and 1% antibiotic solution (GIBCO). All studies were performed on confluent cells of the Caco-2 subclone between passages 40-60.

This epithelial cell line, derived from a human colon adenocarcinoma, was chosen because it also retains the ability for spontaneous differentiation that mimics the normal intestinal ontogeny and epithelial crypt-to –villus migration (Pinto et al., 1983; Briske-Anderson et al., 1997).

For cell viability assessment, once the flasks reached 80% confluence, cells were seeded onto 96 well plates (Corning Costar, NY, USA) at a density of $1\times10^5$ cells/cm$^2$. On day 2, cells were supplemented with different doses of policosanol or octacosanol, using PC (phosphatidyl choline) or BSA (Bovine Serum Albumin) as a carrier to make the micelle. The ratio of policosanol to PC was 1:5, and the concentration of BSA was 0.2% (w/w).

To study policosanol metabolism, once the cells reached 80% confluence, cell cultures were split and seeded onto millicell tissue culture plate inserts (12 mm diameter, 0.4μm pore size, polycarbonate membrane, Corning Costar, NY, USA) at a density of $2\times10^5$ cells/cm$^2$ and placed in six-well culture plates. This permitted a separate access to the luminal (apical) and lymphatic (basolateral) compartments of the monolayers. The apical inserts can hold 0.5 mL media and basolateral plates can hold 2 mL media without spillover. Then, cells were left in culture for 21 days, at which time a highly differentiated phenotype was clearly appreciable (Napolitano et al., 2003). The culture medium was regularly changed once every second day.

On day 21, the basolateral compartment medium was replaced with 2 ml of HEPES/carbonate-buffered EMEM (Earle's Minimum Essential Medium, GIBCO, Grand Island, NY) supplemented with 4% (v/v) Fetal Bovine Serum (GIBCO) and 1% antibiotic-antimycotic solution (GIBCO), and the apical compartment medium was replaced with a fixed policosanol micelle, using PC as a carrier or control micelle.

## 3.5. Cell Count and Light Microscopy

Cells were released by brief EDTA–trypsin treatment and were collected after centrifuging and stained with trypan blue (Sigma-Aldrich Canada Ltd., Oakville Ontario, Canada). Aliquots were taken and counted under the microscope (2.5×10) with a hematocytometer.

## 3.6. Morphological studies

Before the absorption and metabolism study, the Caco-2 cells monolayer was checked by light microscopy and was further processed for scanning electron microscopy (SEM) and Transmission Electron Microscope (TEM) in order to ascertain how Caco-2 cells resemble the small intestinal enterocytes. Caco-2 cells were cultured in the 25-$cm^2$ flasks.

On day 21, the monolayer was assessed by light microscopy, and then cells were collected, prefixed in 2.5 % glutaralderhyde in cacodylate buffer (pH 7.2) for 1.5 hr at room temperature. Cells were washed in the same buffer 3 times for 15 min each time. Cells were post-fixed in 1% osmium in the buffer as above for 1.5 hr at room temperature. Cells were briefly rinsed in distilled water. Cells were dehydrated in a series of 50%, 70%, 90% and absolute ethanol for 10 min in each grade, then for 10 min in each of two additional absolute ethanol. Cells were embedded in Spurr's resin mixture (Spurr's resin/ ethanol 50:50) overnight, and complete Spurr's resin for overnight in a vacuum desiccator. Following polymerization at 70 C for 12 hr, the ultrathin sections were cut and stained in Uranyl acetate and lead citrate and examined with a Hitachi H7000 Electron Microscope (Tokyo, Japan).

Alternatively, on day 21, the monolayer was assessed by normal microscopy, and then prefixed in 2.5 % glutaraldehyde in Minllong's buffer (pH 7.3) for one hour at room temperature. A small piece was cut from the flask to get a sample. The sample was washed in the same buffer for three times, 10 min each time. Then it was postfixed it in 1 % osmium in the same buffer for one hour at room temperature. It was washed in distilled water briefly. The sample was dehydrated in a series of ethanol (50 %, 70 %, 90 % & 100 %), 10 min in each grade, then for 10 min in each of two absolute ethanol washes. The sample was processed via critical point dry at 31 ℃ for 5 min. The sample was mounted on the stub and coated with gold with a sputter coater (Edwards S150B, England). Finally, the sample was examined with a Hitachi Scanning Electron Microscope S2500 (Tokyo, Japan).

## 3.7. Assessment of Cells monolayer integrity

The transepithelial electrical resistance (TEER), as the index of confluence and

integrity of monolayers (Haidalgo et al, 1989; Mehran et al, 1997), was measured on day 21, using an epithelial volt ohmmeter (EVOM) (World Precision Instruments, Hamden, CT, U.S.A.) with a pair of chopstick electrodes. Results were expressed as ohm/cm2.

## 3.8. MTT solution preparation

MTT 〔3-(4,5)dimethylthiazol-2-yl-2,5-diphenyltetrazolim〕 was dissolved in PBS (phosphate buffered saline) to a concentration of 5 mg/ml and filtered sterilized. The solution was stored at 4℃ wrapped in aluminum foil to protect it from light.

## 3.9. MTT assay

Based on Denizot and Lang (1986) method, MTT 〔3-(4,5)dimethylthiazol-2-yl-2,5-diphenyltetrazolim〕 bromide assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of yellow MTT to form purple-blue formazan crystals. The crystals are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Solubilization of cells with the addition of detergent results in liberation and solubilization of the crystal. The number of surviving cells is directly proportional to the amount of purple-blue formazan crystals. The color can then be quantified using a simple colorimetric assay. The result can be read on a multi-well scanning spectrophotometer (ELISA reader).

For the cell viability determination, 10μl of MTT/well was added in a 96-well plate after 48 hours incubation with a different concentration of policosanol micelle or octacosanol micelle, and incubated for 4 hours at 37℃ in an atmosphere of 5% CO2, 95% air. The media were aspirated with a syringe needle (B-D 20G11/2, Bectom Dickinson and company, NJ, USA). The purple formazan crystals were dissolved in 100μl of DMSO (dimethyl sulfoxide, Fisher Scientific, PA, USA). The plate was covered in the dark with aluminum foil at room temperature for about 30 minutes. The absorbance was read at 570 nm with a spectrophotometer (Spectra max 190, Molecular Devices Corporation, California, USA ).

## 3.10. Collection lipid samples from Trans-well Plates.

To collect the lipid sample from trans-well plates treated with policosanol, media

at bottom of each trans-well plates was collected after 48 hours incubation with policosanol micelle and combined together into one 15 ml centrifuge tube (Fisher Scientific, Canada) labeled lymphatic (basolateral) policosanol. Media on top of each control trans-well plate was collected and combined together into one 15 ml centrifuge tube labeled luminal (apical) policosanol. The tubes were centrifuged for 5 minutes at 1,000 rpm (Jouan Centrifuge CR422).The upper clear phase was used for lipid extraction.

To collect the lipid samples from control trans-well plates, the above steps were repeated, except the centrifuge tubes were labeled with control instead of policosanol.

## 3.11. CaCo-2 Cells isolation

To isolate cells from monolayers treated with policosanol, Caco-2 cell monolayers were washed with 0.5 ml of PBS (phosphate buffered saline, Ph 7.4) on the top and 2 ml of PBS at the bottom of the transwell plate incubated with policosanol micelle to remove serum, after the media were collected from both bottom and top compartments. Then the monolayers were washed with 0.5 ml of tris-EDTA wash buffer (0.02% EDTA, 0.8% NaCl, 0.04% KCl, 0.28%Tris HCl and 0.08% Tris Base, Ph 7.15) on top and 2 ml of above buffer at bottom to help loose the cells from insert polycarbonate membrane. And then trypsin-EDTA (0.5 ml, 0.25% ) (Invitrogen Corporation) was added to the top and 2 ml of trypsin-EDTA to the bottom of monolayers. The trans-well plates were kept for 10 minutes in 37 ℃ incubator to facilitate removal of cells inserts into solution. Typsin-EDTA solution was vortexed with 1 ml tip (Fisher Scientific, Canada) on the trans-well plates, collected and combined together into a 50 ml centrifuge tube (Fisher Scientific, Canada) containing 10 ml serum free media labeled policosanol cells. The monolayers were washed twice with serum free media and the wash media were put into the above tube. The tube was centrifuged for 5 minutes at 1,000 rpm (Jouan Centrifuge CR422). The upper clear phase was removed and cells at the bottom saved.

The above steps were repeated to isolate cells from control monolayers.

## 3.12. Cells protein measurement

Tris-mannitol lysis buffer was added to cells isolated from monolayers, and then sonicated for 30 seconds. 0.1 ml of Cells lysate was saved into a 1.5ml microcentrifuge

tube (Fisher Scientific, Canada). This tube was centrifuged at 12,000 rpm (Eppendorf Centrifuge 417C) for 2 minutes and the supernatant was used for BCA (bicinchoninic acid) assay (Pierce Chemical Co.) to measure protein. The rest of the cell lysate was used to extract lipid.

## 3.13. Lipid and policosanol extraction from samples

Following the Folch extraction method (Folch, 1957), all the media and cell samples collected were divided into 25 ml glass tubes (Fisher Scientific, Canada) with black caps (Fisher Scientific, Canada), by 1 ml of sample into each 25 ml tube. Then 20 ml of chloroform:methanol 2:1 was added into each tube. The tubes were shaken overnight at 4℃. Then 3 ml of 0.025% $CaC_{l2}$ was added into each tube. The tubes were vortexed for 30 seconds and left overnight at 4℃ or until the mixture was separated into two phases. The lower lipid containing phase was removed into a 10 ml tube by pipette and evaporated to dryness under stream of nitrogen at 35℃.

## 3.14. Thin-layer Chromatography (TLC) for separation of lipid

Thin layer chromatography plates (20×20cm, 250μm thickness, analtech, Newark, Delaware) were heat activated at 110℃ for 60 minutes after washes in chloroform. TLC tanks lined with Watman #1 filter paper were saturated with the solvent system contained petroleum ether: diethyl ether: glacial acetic acid (80:20:1/v:v:v). Plates were processed 20-25 minutes, or until the solvent front was approximately 3 cm from the top of the plate. A mixture of appropriate lipid standards was used to identify respective lipid bands. Under UV light, lipid bands, containing phospholipids, free cholesterol, policosanol, free fatty acid, triglyceride and cholesterol ester were identified and marked. Each band was scraped into a 6ml glass tube, sealed with Teflon-lined caps and stored at -80℃.

## 3.15. HPK Thin-layer Chromatography (TLC) for separation of tiny lipid

KPK thin layer chromatography plates ( 10×10cm, 200μm thickness, Whatman) were used instead of 20×20cm TLC for the lipid extracted from cells and luminal (apical) compartments using the same method.

## 3.16. Saponification

Cholesteryl ester samples from TLC "G" plates were saponified with 1 ml of 0.5 N methanolic KOH (14.03 g KOH in 500 ml methanol). C19:0 nonadecanoic acid (Sigma Chemical Co.) 2μg internal standard was added. Tubes were capped tightly and heated in heating module (Pierce chemical company, IL, USA) at 110 ℃ for two hours, then cooled 30 minutes before methylation.

For triglyceride, the same method was followed, however samples were heated for one hour instead of two hours.

## 3.17. Methylation

All samples including phospholipids, free fatty acid, triglyceride and cholesteryl ester were methylated using the procedure described by Morrison and Smith (1964). Samples were prepared by adding 1ml of 14% boron triflouride in methanol (w/v) and 2 ml of hexane. An internal standard, 2μg C19:0 nonadecanoic acid (Sigma Chemical Co.) was added to all fractions. Tubes were capped and heated in a heating module at 110℃ for one hour. Once cooled, 1 ml of distilled water was added to wash the sample and then left overnight to separate. The upper hexane layer, containing fatty-acid methyl esters, was removed, concentrated and flushed with nitrogen. Samples were stored at -80℃ until GC analysis.

## 3.18. Gas-Liquid Chromatography of fatty acids

Fatty-acid methyl esters from cholesterol ester, triglyceride, free fatty acids and phospholipids were separated by gas-liquid chromatography. An automated Gas-liquid chromatograph (GLC), Varian Model star 3400X equipped with a Vista 8000 autosampler (Varian Instrument Company, Georgetown, Ontario) was employed. The system utilized a bonded phase fused silica BP20 capillary column (25×0.25mm I.D, SEG International Pty. Ltd). The carrier gas was helium with a flow rate of 1.8 ml/minute using a split ratio of 28:1. The oven temperature operated on four stages. The initial temperature is 90℃, was held for 4 minutes. This was followed by an increase to 170℃, at 25℃/minute, and was held for 13.8 minutes. And then followed by an increase to 190℃, at 5℃/minute, and

was held for 17 minutes. And finally, followed by an increase to 230℃, at 5℃/minute, and was held for 5 minutes, resulting in a total analysis time of 55 minutes. These conditions separated all saturated, mono-, di-, and polyunsaturated fatty acids from 10 to 24 carbons in chain length. A Varian Vista 654 Data System was used to analyze resolved peaks and to quantitate fatty acids. All fatty acid data is expressed as %wt/wt.

## 3.19. Statistical analysis

Data is presented as the mean±S.E.M. Comparison between the lipid profiles of control and policosanol treated cells was made by Student's t-test. Analysis of the effect of policosanol on the growth of Caco-2 was performed by ANOVA and Duncan's multiple range tests (Steel RGD and Torrie JH, 1980). $P<0.05$ was considered to be statistically significant.

# Chapter 4

# RESULTS

## 4.1 The composition and solubility of policosanol

The exact composition of policosanol was tested before being used in all experiments. Policosanol powder was directly analyzed as trimethylsilyl (TMS) derivatives. Alternatively, policosanol powder was dissolved in chloroform and aliquots of solution were evaporated before it was derivated to TMS-ether.

**Table 4.1 Policosanol composition of sugar cane wax extract**

| Individual alcohol | Original powder (% of total mixture) | Chloroform Solution (% of total mixture) |
|---|---|---|
| C24 | 6.9±0.73 | 6.9±0.29 |
| C26 | 9.8±0.43 | 9.7±0.18 |
| C27 | 0.4±0.04 | 0.4±0.08 |
| C28 | 65.7±0.85 | 65.5±0.32 |
| C29 | 0.5±0.02 | 0.5±0.01 |
| C30 | 10.1±1.19 | 10.3±0.26 |
| C32 | 4.9±0.65 | 4.9±0.20 |
| C34 | 1.8±1.30 | 1.9±0.28 |

Values are means±S.E.M., n=4 and are expressed the percentage of total mixture. C24=tetracosanol, C26=hexacosanol, C27=heptacosanol, C28= octacosanol, C29=nonacosanol, C30=triacontanol, C32=dotriacontanol, C34= tetratriacontanol.

The results （Table 4.1） showed that the major components of policasonal are octacosanol, triacontanol and hexacosanol with tetracosanol, heptacosanol, nonacosanol, dotriacontanol and tetratriacontanol existing as minor components. The mixture is similar to other reports. For example, Menendez et at found that policosanol contains: octacosanol (C28) (60–70%), hexacosanol (C26) (3.0–10.0%), triacontanol (C30) (10.0–15.0%) and dotriacontanol (C32) (5.0–10.0%), etc (Menendez et al, 2005).

There is no significant difference between the two methods, suggesting that the

very long chain fatty alcohols are similarly soluble in chloroform, so they have similar solubility characteristics.

The uptake and transport of an ingredient is relevant to the basic characteristics of the compound. To further learn the characteristics of policosanol, especially the solubility, several common solvents were used. Firstly, policosanol over-saturated solutions were made in different solvents, and then the solutions were filtered via filter paper (fisher filter paper, diameter: 5.5 cm, medium porosity). Aliquots of filtered solution were dried in the oven at 80℃ for 12 hours or until the weight was constant. The solubility was calculated as following formula:

Solubility = (Final weight of tube – original weight)/aliquots of over-saturated filtered solution.

**Table 4.2 Solubility of policosanol in different solvents (mg/ml)**

| Chloroform | Chloroform/Methanol | Hexane | Ethanol | Water |
|---|---|---|---|---|
| 11.1±0.25 | 8.3±0.37 | 0.9±0.13 | 0.7±0.06 | 0.00 |

Data represent mean±SEM n=4, at indoors condition.

Policosanol has the highest solubility in chloroform (11.1±0.25) mg/ml, greater than the solubility in chloroform/methanol mixture. Policosanol dissolve poorly in ethanol (0.7 mg/ml) (Table 4.2). Policosanol is not soluble in water, suggesting that a special carrier is necessary to deliver policosanol into aqueous media in the following experiments.

## 4.2 The effect of policosanol/octacosanol on Caco-2 proliferation.

### 4.2.1 Binding of octacosanol to PC and BSA

Phosphatidylcholine (PC) and bovine serum albumin (BSA) were used as the carrier in the preparation of micelles, as neither policosanol nor octacosanol is soluble in aqueous media. The carrier abilities of PC and BSA to octacosanol were studied.

Octacosanol 1.14 μg was extracted from 2 ml of PC micelle (table 4.3), while the concentration of PC used in the micelle is 2 mg/ml. PC carrier ability for octacosanol is 1.14/2/2=0.29 μg/mg. Similarly, binding of octacosanol to BSA is 2.97/2/2=0.75 μg/mg. The PC and BSA carrier ability for policosanol is about 0.29 μg/mg and 0.75μg/mg, respectively, since these very long chain fatty alcohols have similar solubility characteristics.

**Table 4.3 Binding ability of PC and BSA to octacosanol**

| Octacosanol extract from 2 ml of 2mg/ml PC micelle (μg) | Octacosanol extract from 2 ml of 2mg/ml BSA micelle (μg) |
|---|---|
| 1.14±0.04 | 2.97±0.10 |

Data represent the mean±SEM, n=4.

### 4.2.2 The inhibitory effect of policosanol/octacosanol on Caco-2 growth

Cell viability study was conducted before the absorption and metabolism study. To examine the effect of very long chain alcohols on the growth of Caco-2 cell, policosanol was studied using PC as a carrier to deliver policosanol to Caco-2 cells, based on the solubility characteristics of policosanol.

Policosanol will inhibit the proliferation of Caco-2 cell, when PC is used as a carrier (Fig. 4.1). This inhibitory effect is policosanol dose-dependent, starting from 1.14μg/ml base on 48 hours incubation. Thus, the policosanol metabolism study was carried out with 0.57 μg/ml policosanol, at which concentration cell viability is not significantly affected.

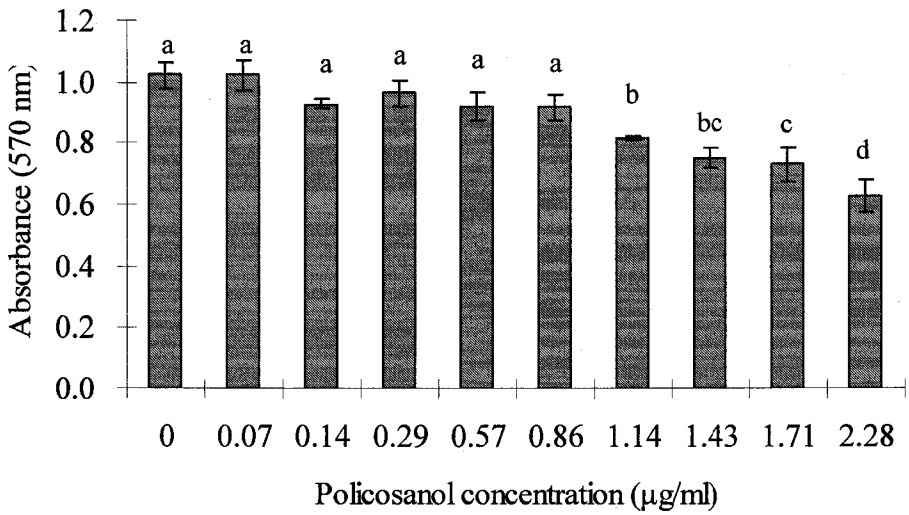


**Figure 4.1 Inhibitory effect of policosanol on the growth of Caco-2 cells using PC as a carrier.**

Caco-2 cells were seeded at a density of 1×105 cells/ cm2 in 96-well plates in HEPES/carbonate-buffered EMEM supplemented with 10 % FBS and 1% antibiotic. After 24 hours, media were changed and incubated with 4% FBS medium and increasing concentration of policosanol, while the concentration of PC maintains same. Cell numbers were determined by the MTT after 48 hours incubation. Data is mean±SEM, n=3; PC: phosphatidylcholine. Bars with different letter means significant difference at $p<0.05$

To further examine the inhibitory effect of policosanol on the growth of Caco-2, BSA (Bovine serum albumin) was used as a carrier when making the policosanol micelle. The concentration of BSA is 0.2% (w/w).

Policosanol will also inhibit the proliferation of Caco-2 cells, when BSA is used as a carrier, starting from 0.05μg/ml of policosanol after 48 hours incubation (Fig. 4.2). This inhibitory effect seems to not be correlated to the dose of policosanol.

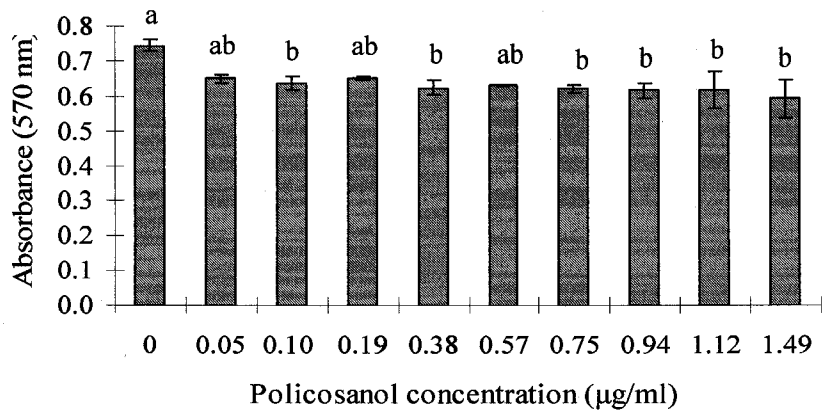


**Figure 4.2 Inhibitory effect of policosanol on the growth of Caco-2 cells using BSA as a carrier.**

Caco-2 cells were seeded at a density of 1×105 cells/ cm2 in 96-well plates in HEPES/carbonate-buffered EMEM supplemented with 10 % FBS and 1% antibiotic. After 24 hours, media were changed and incubated with 4% FBS medium and increasing concentration of policosanol, while the concentration of BSA maintains same, 0.2% (w/w). Cell numbers were determined by the MTT after 48 hours incubation. Data is mean±SEM, n=3; BSA: Bovine Serum Albumin. Bars with different letter means significant difference at $p<0.05$.

As octacosanol is the major species of policosanol, it was used to examine the inhibitory effect of very long chain fatty alcohols on the Caco-2 cell proliferation. Octacosanol accounts for approximately 66% of the mixture of very long chain fatty alcohols.

Octacosanol alone can inhibit proliferation of Caco-2 cells (Fig. 4.3), when BSA is used as a carrier. Similar with policosanol, the inhibitory effect starts from 0.05μg/ml of octacosanol after 48 hours incubation. However, octacosanol failed to further inhibit when the concentration was increased to over 1.12 μg/ml.

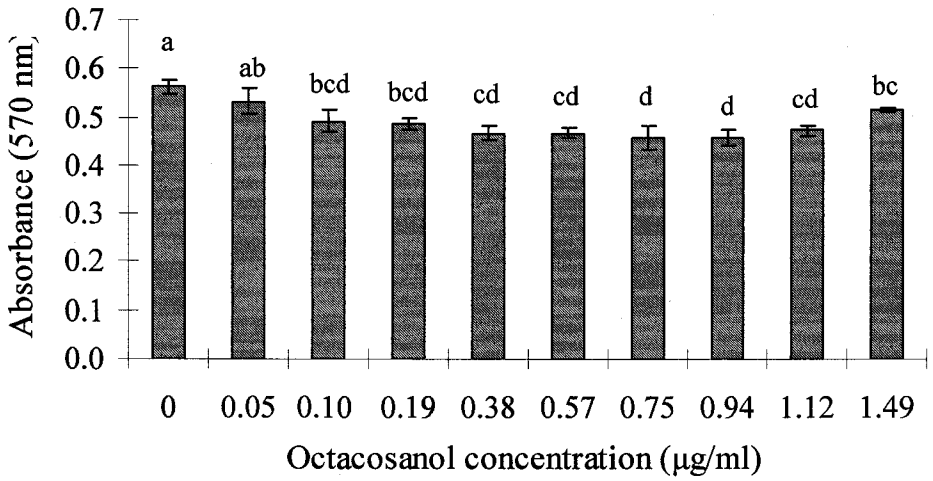


**Figure 4.3 Inhibitory effect of octacosanol on the growth of Caco-2 cells using BSA as a carrier.**

Caco-2 cells were seeded at a density of 1×105 cells/ cm2 in 96-well plates in HEPES/carbonate-buffered EMEM supplemented with 10 % FBS and 1% antibiotic. After 24 hours, media were changed and incubated with 4% FBS medium and increasing concentration of octacosanol, while the concentration of BSA maintains same, 0.2% (w/w). Cell numbers were determined by the MTT after 48 hours incubation. Data are means±SEM, n=3; BSA: Bovine Serum Albumin. Bars with different letter means significant difference at $p<0.05$.

## 4.3. Characterization of Caco-2 cells monolayer

The morphology of a typical monolayer of Caco-2 cells cultured in 75cm$^2$ was evaluated on day 21 by light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Fig. 4.4). As can be observed, Caco-2 cells proliferated and differentiated into a well-polarized monolayer with tight junctions and brush border microvilli on the surface. The single cell resembles small intestinal enterocytes.

To assess the cell monolayer integrity, transepithelial electrical resistance (TEER) across tissue culture plate inserts was evaluated by epithelial volt ohmmeter (EVOM). At day 21, this parameter is 1070±36.7 Ω /cm$^2$, suggesting the Caco-2monolayer is ready to conduct absorption and metabolism. This result is similar with the other reports, such as: Napolitano et al (2003) who found 930±6.64Ω/ cm$^2$, and Marcil et al (2003) who found 1211±63 Ω /cm$^2$. The monolayer was excluded, if TEER was lower than 800 Ω /cm$^2$.

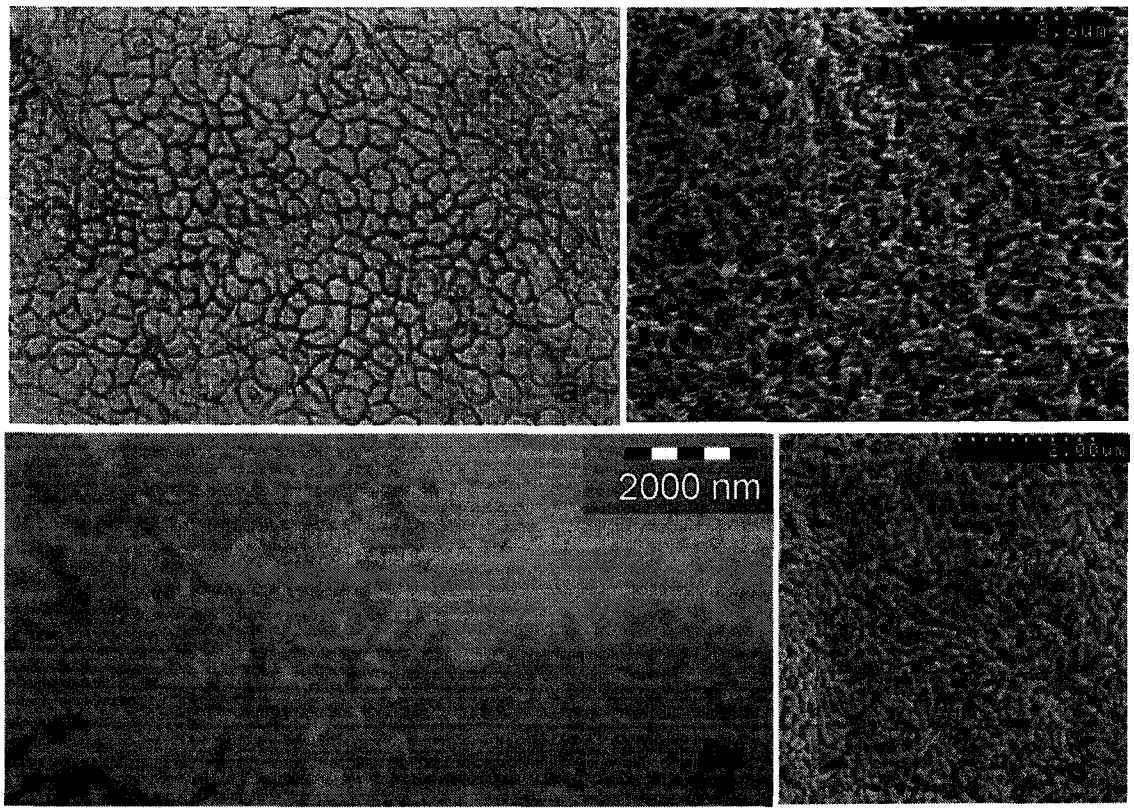


**Figure 4.4 Micrographs of the Caco-2 cells monolayer on day 21 of culture.**

As can be seen, cells show morphological characteristics resembling those of small intestinal enterocytes. A well-polarized columnar body with tight junctions (a) (under light microscope) and typical brush border microvilli projecting to the cell surface was evident (b, d) (under SEM). A single Caco-2 cell also shows microvilli (c) (under TEM). Magnifications: (a) 25×, (b), (c) and (d) are showed on the scales.

## 4.4. The composition of policosanol after 48 hours incubation in the Caco-2 cells monolayer

To test policosanol constituent change after absorption by the Caco-2 cells monolayer, the apical media was replaced with 0.57 μg /ml of policosanol and PC micelle in the apical compartments on day 21. From the effect of policosanol on the growth of Caco-2 cells study, this concentration of policosanol will not affect the Caco-2 cells

viability. There is some policosanol left after 48 hours incubation in the Caco-2 cells monolayer. The policosanol composition profile does not change significantly before incubation and that after incubation (Table 4.4). The slight difference in C29 (nonacosanol) and C32 (dotriacontanol) maybe result from free fatty acids in the fetal bovine serum (FBS). Apparently, each long chain alcohol has similar overall absorption characteristics.

When pure octacosanol was tested, no other very long chain fatty alcohols were found (Data is not shown), suggesting that policosanol/octacosanol will not been degraded or elongated into other very long chain fatty alcohols.

**Table 4.4 Policosanol constituent change after 48 hours incubation**

| Individual alcohol | Original powder (% of total mixture) | After 48 hours incubation (% of total mixture) |
|---|---|---|
| C24 | 6.9±0.73 | 6.4±0.28 |
| C26 | 9.8±0.43 | 9.3±0.51 |
| C27 | 0.4±0.04 | 0.3±0.29 |
| C28 | 65.7±0.85 | 65.5±1.61 |
| C29 | 0.5±0.02 | 0.2±0.22 |
| C30 | 10.1±1.19 | 10.0±0.07 |
| C32 | 4.9±0.65 | 6.2±0.54 |
| C34 | 1.8±1.30 | 2.2±0.83 |

Values are means±S.E.M., n=3 and are expressed the percentage of total mixture. C24=tetracosanol, C26= hexacosanol, C27= heptacosanol, C28= octacosanol, C29= nonacosanol, C30=triacontanol, C32= dotriacontanol, C34= tetratriacontanol.

## 4.5 Absorption of octacosanol after 48 hours incubation of Caco-2 monolayer

To study the absorption of very long chain fatty alcohols, media both in the apical and basolateral compartments of monolayer as well as cells were collected and extracted after 48 hours incubation of the Caco-2 cells monolayer. There was no apparent

policosanol/octacosanol found in the basolateral compartment media under UV light (UVGL-25, upland, CA, USA). There is no visible policosanol/octacosanol intracellular accumulation in the cells under UV light. The octacosanol amount left in the apical compartment is 0.24μg after 48 hours incubation. While, 3 ml of octacosanol micelle was added into the apical compartment (6 dishes), that will deliver 0.57*3=1.71μg of octacosanol (Fig.4.4). The octacosanol absorption is (1.71-0.24)/1.7=86.0% based on 48 hours incubation.

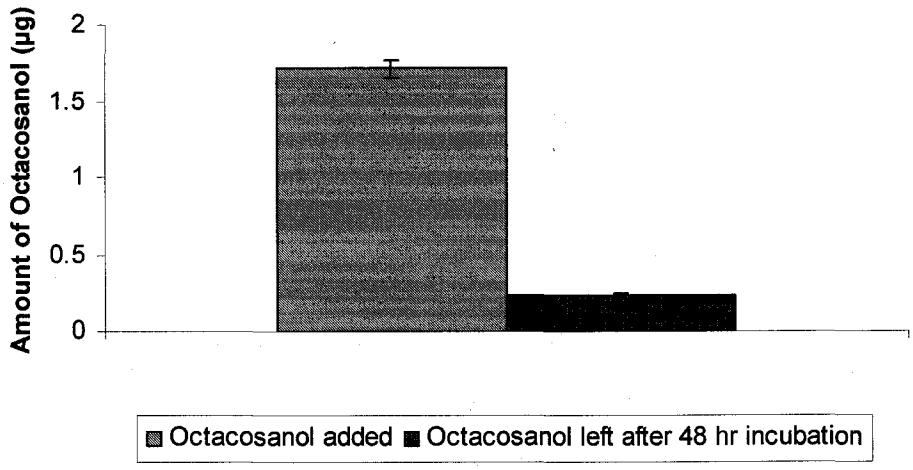


**Figure 4.5 Absorption rate of octacosanol in Caco-2 cells monolayer**
Octacosanol micelle was added into apical compartment at day 21, and the apical media were collected after 48 hours incubation to assess how much octacosanol was left. Data is mean±SEM, n=4.

## 4.6 Effect of policosanol treatment on fatty acids amount

To evaluate whether policosanol changed the lipid fatty acids of cells and media after being absorbed, both the luminal (apical) and the lymphatic (basolateral) medium as well as cells were collected and extracted. Fatty acids amounts in cholesteryl ester, triglycerides, free fatty acids and phospholipids were quantified.

The incubation of fully differentiated Caco-2 cells with policosanol significantly increased the secretion of the fatty acids in cholesteryl ester and the secretion of free fatty acids into the lymphatic (basolateral) medium. Policosanol also decreased secretion of fatty acids in triglycerides from 0.45±0.03 μg/ml to 0.38±0.02 μg/ml. This difference did not reach statistical significance. Policosanol did not affect the amount of fatty acid in the

phospholipids examined (Table 4.5).

**Table 4.5 Effect of policosanol treatment on fatty acids amount in basolateral (lymphatic) medium**

| | Control (μg /ml) | Policosanol(μg /ml) |
|---|---|---|
| CE | 1.4±0.07 | 1.7±0.09* |
| TG | 0.45±0.03 | 0.38±0.02 |
| FFA | 0.42±0.09 | 0.64±0.19* |
| PL | 2.0±0.10 | 2.0±0.14 |

Values represent means±S.E.M. expressed as fatty acid amount of lipid (μg /ml). n=3. CE: cholesteryl ester; TG: triglycerides; FFA: Free fatty acids: PL: phospholipids.
Significant difference between control and policosanol medium: *$p<0.05$.

Policosanol significantly increased the amount of cholesteryl ester fatty acids and decreased the free fatty acid amount in Caco-2 cells. Policosanol did not change the fatty acids amount in triglyceride and phospholipids in Caco-2 cells (table 4.6).

**Table 4.6 Effect of policosanol treatment on fatty acid amount in Caco-2 cells**

| | Control (μg /mg protein) | Policosanol (μg /mg protein) |
|---|---|---|
| CE | 4.2±0.13 | 5.0±0.28* |
| TG | 123.6±11.9 | 127.0±11.4 |
| FFA | 14.9±0.75 | 11.8±0.82* |
| PL | 77.0±3.7 | 76.1±3.5 |

Values represent means±S.E.M. expressed as fatty acid amount of lipid per mg protein (μg /mg). n=3. CE: cholesteryl ester; TG: triglycerides; FFA: Free fatty acids: PL: phospholipids. Significant difference between control and policosanol treated cells *$p<0.05$.

Policosanol significantly decreased the efflux of fatty acid in cholesteryl ester into the luminal (apical) medium. Policosanol also slightly deceased the efflux of fatty acid amount of the triglycerides from 1.7±0.11 μg/ml to 1.2±0.14 μg/ml, although this difference did not reach statistical significance. Policosanol did not change the fatty acid

amount in phospholipids in the medium (table 4.7).

**Table 4.7 Effect of policosanol treatment on amount of fatty acids in apical (luminal) medium**

| | Control (μg/ml) | Policosanol (μg/ml) |
|---|---|---|
| CE | 3.2±0.11 | 2.6±0.19* |
| TG | 1.7±0.11 | 1.2±0.14 |
| FFA | N/A | N/A |
| PL | 313.5±19.7 | 308.9±14.3 |

Values represent means±S.E.M. and are expressed as fatty acid amount of lipid (μg /ml). n=3. CE: cholesteryl ester; TG: triglycerides; FFA: Free fatty acids; PL: phospholipids. N/A: not available. Significant difference between control and policosanol treated medium*$p<0.05$.

## 4.7 Effect of policosanol treatment on fatty acids profiles

To evaluate whether policosanol changed the fatty acid profiles of lipid in cells and media after being absorbed, both the medium in the luminal (apical) and in the lymphatic (basolateral) compartments as well as in the cells were collected and extracted. Fatty acids content in cholesteryl ester, triglycerides, and phospholipids was quantified.

Policosanol significantly increased the n-6 polyunsaturated fatty acids (Table 4.8) from 11.0% to 13.1% into the cholesterol ester in the lymphatic (basolateral) medium. The trend of the increasing n-6 fatty acid content include: linoleic acid (18:2n-6), gamma linoleic acid (18:3n-6), dinomo gamma linoleic acid (20:3n-6) and arachidonic acid (20:4n-6) with corresponding slightly decreasing content of lauric acid (C12) and myristic acid (C14) (Table 4.8).

Policosanol significantly increased the proportion of behenic acid (C22:0) from 0.7% to 2.0% into the phospholipids in the lymphatic (basolateral) medium and corresponded to the slightly decreasing content of palmitic acid (C16) (Table 4.9).

Policosanol did not significantly modulate the fatty acid profile in triglycerides or phospholipids of Caco-2 cell (Tables 4.10 and 4.11).

Policosanol did not significantly modulate the fatty acid profiles of cholesterol ester or phospholipids in the luminal (apical) medium (table 4.12 and table 4.13).

**Table 4.8 Effect of policosanol treatment on cholesterol ester fatty acid composition in the lymphatic (basolateral) medium**

| | Control medium | Policosanol medium |
|---|---|---|
| C10:0 | 0.2±0.10 | 0.1±0.05 |
| C12:0 | 0.3±0.11 | 0.1±0.03 |
| C14:0 | 3.4±0.38 | 2.4±0.44 |
| C14:1n-5 | 0.4±0.14 | 0.4±0.11 |
| C15:0 | 2.4±0.48 | 1.8±0.26 |
| C16:0 | 33.9±1.82 | 33.1±1.49 |
| C16:1n-7 | 5.4±0.32 | 5.7±0.34 |
| C17:1n-8 | 1.3±0.37 | 1.1±0.44 |
| C18:0 | 5.7±0.59 | 6.0±0.33 |
| C18:1n-9 | 25.6±1.12 | 26.1±1.48 |
| C18:1n-7 | 8.0±0.42 | 8.1±0.38 |
| C18:2n-6 | 7.8±0.59 | 8.7±0.90 |
| C18:3n-6 | 0.4±0.28 | 0.5±0.41 |
| C18:3n-3 | 0.1±0.04 | 0.1±0.04 |
| C20:0 | 0.4±0.11 | 0.4±0.14 |
| C20:1n-9 | 0.3±0.07 | 0.4±0.16 |
| C20:2n-6 | 0.2±0.11 | 0.2±0.10 |
| C20:3n-6 | 0.4±0.12 | 0.7±0.23 |
| C20:4n-6 | 2.1±0.33 | 2.8±0.42 |
| C20:3n-3 | 0.1±0.04 | 0.03±0.02 |
| C20:5n-3 | 0.1±0.08 | 0.02±0.02 |
| C22:0 | 0.02±0.02 | 0.2±0.16 |
| C22:1n-9 | 0.2±0.12 | 0.5±0.47 |
| C22:2n-6 | 0 | 0.01±0.01 |
| C22:4n-6 | 0 | 0.1±0.07 |
| C22:5n-6 | 0.1±0.05 | 0.1±0.04 |
| C22:6n-3 | 0.3±0.09 | 0.4±0.07 |
| C24:0 | 0.3±0.09 | 0.2±0.06 |
| C24:1n-9 | 0.6±0.35 | 0.1±0.11 |
| ∑SAT | 46.7±2.30 | 44.2±2.17 |
| ∑MUFA | 41.8±1.94 | 42.3±2.24 |
| ∑n-6 PUFA | 11.0±0.70 | 13.1±0.56* |
| ∑n-3 PUFA | 0.6±0.13 | 0.5±0.11 |
| SAT/UNSAT | 0.9±0.08 | 0.8±0.08 |
| n-6/n-3 | 19.8±4.12 | 27.1±2.74 |

Values represent means±S.E.M. and are expressed as molar percentages of total fatty acids. n=3. ∑SAT: total saturated fatty acids; ∑MUFA: total monounsaturated fatty acids; ∑n-6 PUFA: total n-6 polyunsaturated fatty acids; ∑n-3 PUFA: total n-3 polyunsaturated fatty acids. UNSAT: total unsaturated fatty acids;
Significant difference between control and policosanol treated medium *$p<0.05$.

**Table 4.9 Effect of policosanol treatment on phospholipids fatty acid composition in the lymphatic (basolateral) medium**

| | Control medium | Policosanol medium |
|---|---|---|
| C10:0 | 0.2±0.16 | 0.04±0.04 |
| C12:0 | 0.1±0.06 | 0.3±0.21 |
| C14:0 | 0.4±0.23 | 0.6±0.24 |
| C14:1n-5 | 0.4±0.21 | 0.6±0.46 |
| C15:0 | 0.2±0.20 | 0.7±0.38 |
| C16:0 | 27.1±1.71 | 23.9±1.90 |
| C16:1n-7 | 0.9±0.48 | 0.4±0.07 |
| C17:1n-8 | 1.5±1.31 | 0.2±0.09 |
| C18:0 | 23.7±1.46 | 22.8±2.30 |
| C18:1n-9 | 15.4±1.00 | 15.4±0.94 |
| C18:1n-7 | 4.7±0.47 | 5.1±0.55 |
| C18:2n-6 | 5.6±0.48 | 5.7±1.32 |
| C18:3n-6 | 0.4±0.23 | 0.6±0.42 |
| C18:3n-3 | 0.1±0.13 | 1.3±1.32 |
| C20:0 | 0.9±0.21 | 0.8±0.33 |
| C20:1n-9 | 0.6±0.25 | 0.7±0.25 |
| C20:2n-6 | 0.2±0.18 | 0.9±0.40 |
| C20:3n-6 | 1.0±0.27 | 1.7±0.64 |
| C20:4n-6 | 3.9±0.09 | 4.5±0.87 |
| C20:3n-3 | 0.1±0.07 | 0.1±0.09 |
| C20:5n-3 | 0 | 0.1±0.05 |
| C22:0 | 0.7±0.33 | 2.0±0.40* |
| C22:1n-9 | 0.04±0.04 | 0.2±0.19 |
| C22:2n-6 | 0.1±0.09 | 0 |
| C22:4n-6 | 0.4±0.16 | 0.2±0.18 |
| C22:5n-6 | 2.4±0.22 | 2.4±0.31 |
| C22:6n-3 | 3.0±0.21 | 2.7±0.39 |
| C24:0 | 2.0±0.67 | 2.6±0.62 |
| C24:1n-9 | 4.1±0.52 | 3.8±0.62 |
| ∑SAT | 55.3±0.91 | 53.7±1.39 |
| ∑MUFA | 27.6±1.26 | 26.4±0.74 |
| ∑n-6 PUFA | 13.9±0.81 | 15.9±2.03 |
| ∑n-3 PUFA | 3.2±0.31 | 4.1±1.01 |
| SAT/UNSAT | 1.3±0.03 | 1.2±0.07 |
| n-6/n-3 | 4.5±0.37 | 4.9±1.25 |

Values represent means±S.E.M., for further details see Table 4.8. Significant difference between control and policosanol treated medium *$p<0.05$. . n=3

**Table 4.10 Effect of policosanol treatment on triglyceride fatty acid composition in Caco-2 cells**

| | Control Cells | Policosanol treated cells |
|---|---|---|
| C10:0 | 0 | 0 |
| C12:0 | 0.1±0.05 | 0.1±0.06 |
| C14:0 | 1.8±0.54 | 2.1±0.48 |
| C14:1n-5 | 0.3±0.11 | 0.5±0.34 |
| C15:0 | 0.6±0.08 | 0.7±0.08 |
| C16:0 | 26.1±2.32 | 25.6±2.12 |
| C16:1n-7 | 2.7±0.37 | 2.8±0.45 |
| C17:1n-8 | 0.7±0.13 | 0.6±0.13 |
| C18:0 | 14.8±0.61 | 14.7±0.76 |
| C18:1n-9 | 24.8±2.60 | 24.1±2.96 |
| C18:1n-7 | 8.4±0.55 | 7.9±0.57 |
| C18:2n-6 | 4.5±0.66 | 4.5±0.79 |
| C18:3n-6 | 0.03±0.02 | 0.04±0.03 |
| C18:3n-3 | 0.8±0.26 | 0.9±0.37 |
| C20:0 | 3.1±1.09 | 3.5±0.94 |
| C20:1n-9 | 1.6±0.13 | 1.5±0.13 |
| C20:2n-6 | 0.3±0.09 | 0.5±0.12 |
| C20:3n-6 | 0.5±0.17 | 0.6±0.23 |
| C20:4n-6 | 1.5±0.32 | 1.5±0.61 |
| C20:3n-3 | 0.1±0.05 | 0.1±0.02 |
| C20:5n-3 | 0.2±0.05 | 0.2±0.05 |
| C22:0 | 1.1±0.36 | 1.4±0.38 |
| C22:1n-9 | 0.4±0.16 | 0.3±0.09 |
| C22:2n-6 | 0.1±0.07 | 0.2±0.09 |
| C22:4n-6 | 0.5±0.10 | 0.4±0.14 |
| C22:5n-6 | 1.2±0.35 | 1.2±0.42 |
| C22:6n-3 | 2.6±0.41 | 2.9±0.56 |
| C24:0 | 0.9±0.35 | 1.0±0.39 |
| C24:1n-9 | 0.4±0.09 | 0.3±0.06 |
| ∑SAT | 48.5±3.17 | 49.1±3.27 |
| ∑MUFA | 39.3±2.66 | 38.0±2.66 |
| ∑n-6 PUFA | 8.7±1.49 | 8.9±2.13 |
| ∑n-3 PUFA | 3.6±0.42 | 4.0±0.74 |
| SAT/UNSAT | 1.0±0.13 | 1.0±0.14 |
| n-6/n-3 | 2.6±0.47 | 2.7±0.72 |

Values represent means±S.E.M., for further details see Table 4.8. n=3. No significant difference between control and policosanol treated medium.

**Table 4.11 Effect of policosanol treatment on phospholipids fatty acid composition in Caco-2 cells**

| | Control Cells | Policosanol treated cells |
|---|---|---|
| C10:0 | 0 | 0 |
| C12:0 | 0.1±0.03 | 0.02±0.02 |
| C14:0 | 1.1±0.33 | 0.8±0.08 |
| C14:1n-5 | 0.2±0.14 | 0.1±0.02 |
| C15:0 | 0.5±0.16 | 0.5±0.10 |
| C16:0 | 23.6±1.69 | 23.4±1.43 |
| C16:1n-7 | 2.8±0.22 | 2.8±0.12 |
| C17:1n-8 | 1.0±0.54 | 0.8±0.12 |
| C18:0 | 19.6±1.11 | 19.6±1.16 |
| C18:1n-9 | 13.8±0.21 | 13.8±0.62 |
| C18:1n-7 | 4.3±0.13 | 4.3±0.27 |
| C18:2n-6 | 10.9±0.63 | 11.3±0.66 |
| C18:3n-6 | 0.01±0.01 | 0.01±0.01 |
| C18:3n-3 | 0.5±0.13 | 0.4±0.08 |
| C20:0 | 0.9±0.09 | 0.8±0.21 |
| C20:1n-9 | 0.5±0.01 | 0.5±0.04 |
| C20:2n-6 | 0.7±0.16 | 0.6±0.16 |
| C20:3n-6 | 2.0±0.21 | 1.9±0.27 |
| C20:4n-6 | 9.4±1.81 | 9.7±2.02 |
| C20:3n-3 | 0.2±0.12 | 0.1±0.03 |
| C20:5n-3 | 0.8±0.19 | 1.4±0.56 |
| C22:0 | 1.0±0.41 | 1.0±0.34 |
| C22:1n-9 | 0.1±0.08 | 0.1±0.06 |
| C22:2n-6 | 0.1±0.05 | 0.04±0.02 |
| C22:4n-6 | 0.5±0.12 | 0.4±0.14 |
| C22:5n-6 | 1.0±0.25 | 1.0±0.20 |
| C22:6n-3 | 2.5±0.40 | 2.7±0.59 |
| C24:0 | 1.3±0.22 | 1.4±0.14 |
| C24:1n-9 | 0.7±0.13 | 0.6±0.10 |
| ∑SAT | 48.1±3.07 | 47.4±2.81 |
| ∑MUFA | 23.4±0.34 | 23.1±0.58 |
| ∑n-6 PUFA | 24.6±2.91 | 24.9±3.29 |
| ∑n-3 PUFA | 4.0±0.38 | 4.6±1.10 |
| SAT/UNSAT | 1.0±0.11 | 0.9±0.10 |
| n-6/n-3 | 6.3±0.54 | 6.7±1.80 |

Values represent means±S.E.M., for further details see Table 4.8. n=3. No significant difference between control and policosanol treated medium.

**Table 4.12 Effect of policosanol treatment on cholesterol ester fatty acid composition in the luminal (apical) medium**

| | Control medium | Policosanol medium |
|---|---|---|
| C10:0 | 0.5±0.32 | 0 |
| C12:0 | 0.9±0.62 | 0.7±0.54 |
| C14:0 | 3.2±0.32 | 2.9±0.68 |
| C14:1n-5 | 0.3±0.14 | 0.4±0.18 |
| C15:0 | 2.1±0.31 | 1.7±0.15 |
| C16:0 | 40.3±2.67 | 35.3±3.58 |
| C16:1n-7 | 3.6±0.73 | 4.4±0.81 |
| C17:1n-8 | 1.5±0.53 | 1.8±0.32 |
| C18:0 | 8.1±1.13 | 7.0±0.67 |
| C18:1n-9 | 17.9±2.23 | 20.9±2.28 |
| C18:1n-7 | 5.3±0.80 | 6.7±0.57 |
| C18:2n-6 | 5.1±0.96 | 6.4±1.26 |
| C18:3n-6 | 0.6±0.47 | 0.9±0.81 |
| C18:3n-3 | 0.2±0.14 | 0.4±0.29 |
| C20:0 | 0.2±0.16 | 0.4±0.20 |
| C20:1n-9 | 0.5±0.31 | 0.6±0.41 |
| C20:2n-6 | 0.1±0.06 | 0.01±0.01 |
| C20:3n-6 | 0.5±0.35 | 0.5±0.24 |
| C20:4n-6 | 1.5±1.03 | 2.2±0.93 |
| C20:3n-3 | 0.2±0.10 | 0.3±0.21 |
| C20:5n-3 | 0.2±0.21 | 0 |
| C22:0 | 0.2±0.12 | 0 |
| C22:1n-9 | 0.5±0.22 | 0.7±0.36 |
| C22:2n-6 | 0.8±0.56 | 0.4±0.30 |
| C22:4n-6 | 0.9±0.85 | 0.5±0.54 |
| C22:5n-6 | 1.0±0.88 | 1.0±0.98 |
| C22:6n-3 | 1.5±1.00 | 1.1±0.31 |
| C24:0 | 1.2±0.66 | 1.1±0.65 |
| C24:1n-9 | 1.3±0.51 | 1.5±0.30 |
| ∑SAT | 56.6±4.30 | 49.1±4.33 |
| ∑MUFA | 30.9±3.73 | 36.9±4.09 |
| ∑n-6 PUFA | 10.5±2.98 | 12.1±2.65 |
| ∑n-3 PUFA | 2.1±1.18 | 1.8±0.44 |
| SAT/UNSAT | 1.4±0.24 | 1.0±0.20 |
| n-6/n-3 | 10.6±2.95 | 8.8±3.03 |

Values represent means±S.E.M., for further details see Table 4.8. n=3. No significant difference between control and policosanol treated medium.

**Table 4.13 Effect of policosanol treatment on phospholipids fatty acid composition in the luminal (apical) medium**

| | Control medium | Policosanol medium |
|---|---|---|
| C10:0 | 0.3±0.26 | 0.2±0.18 |
| C12:0 | 0.2±0.07 | 0.2±0.11 |
| C14:0 | 2.1±1.02 | 1.2±0.48 |
| C14:1n-5 | 7.5±4.22 | 7.5±4.20 |
| C15:0 | 1.8±0.87 | 1.2±1.02 |
| C16:0 | 41.7±2.72 | 38.1±3.00 |
| C16:1n-7 | 0.3±0.05 | 0.2±0.03 |
| C17:1n-8 | 0.1±0.05 | 0.1±0.03 |
| C18:0 | 8.9±1.31 | 7.9±1.11 |
| C18:1n-9 | 12.9±0.76 | 12.5±1.33 |
| C18:1n-7 | 2.4±0.19 | 2.4±0.20 |
| C18:2n-6 | 18.5±4.36 | 21.7±6.15 |
| C18:3n-6 | 0.2±0.13 | 0.04±0.03 |
| C18:3n-3 | 0.5±0.13 | 0.9±0.33 |
| C20:0 | 0.5±0.28 | 0.5±0.39 |
| C20:1n-9 | 0.2±0.06 | 0.1±0.06 |
| C20:2n-6 | 0.03±0.02 | 0.1±0.05 |
| C20:3n-6 | 0.04±0.04 | 0.7±0.68 |
| C20:4n-6 | 0.1±0.07 | 0.04±0.02 |
| C20:3n-3 | 0.00 | 0.01±0.01 |
| C20:5n-3 | 0.04±0.02 | 0.1±0.07 |
| C22:0 | 0.3±0.18 | 0.4±0.15 |
| C22:1n-9 | 0.2±0.09 | 0.3±0.05 |
| C22:2n-6 | 0.1±0.07 | 0.1±0.03 |
| C22:4n-6 | 0.2±0.10 | 0.03±0.02 |
| C22:5n-6 | 0.4±0.14 | 0.2±0.12 |
| C22:6n-3 | 0.3±0.15 | 0.2±0.16 |
| C24:0 | 0.3±0.09 | 1.7±1.62 |
| C24:1n-9 | 0.2±0.10 | 1.4±1.35 |
| ∑SAT | 56.2±3.52 | 51.4±3.80 |
| ∑MUFA | 23.6±3.27 | 24.4±3.19 |
| ∑n-6 PUFA | 19.4±4.38 | 22.9±5.91 |
| ∑n-3 PUFA | 0.8±0.12 | 1.3±0.35 |
| SAT/UNSAT | 1.3±0.17 | 1.1±0.15 |
| n-6/n-3 | 24.5±4.83 | 19.4±1.61 |

Values represent means±S.E.M., for further details see Table 4.8. . n=3. No significant difference between control and policosanol treated medium.

# Chapter 5

# CONCLUSION AND DISCUSSION

## 5.1 CONCLUSION

The hypotheses tested in the thesis have been addressed as follows:

**Hypothesis 1:** Policosanol can be absorbed by Caco-2 cells and will be oxidized and degraded into short-chain fatty acids.

Caco-2 cells viability study demonstrated that policosanol will not affect cells viability at concentration of 0.57 μg/ml. Morphological studies indicated that Caco-2 cells will spontaneously differentiate into an ileal-like polarized cell monolayer with tight junction after 21 days incubation. The cell surface is full of typical brush border microvilli. The single cell resembles the small intestinal enterocytes. Thus the Caco-2 cell monolayer is a suitable model to study the absorption and metabolism of very long chain fatty alcohols in the intestine. The incubation of the Caco-2 cells monolayer with policosanol or octacosanol micelle indicates that octacosanol and policosanol can be absorbed. The octacosanol absorption rate is 86.0% after 48 hours incubation in the Caco-2 cells monolayer. The absorption from the apical side of the Caco-2 cells monolayer in the present study was estimated by analyzing the recoverable policosanol in terms of disappearance from apical media.

Further lipid content evaluation indicated that policosanol significantly increased the cholesteryl ester fatty acids amount both in the cells and lymphatic medium, while significantly decreased the cholesteryl ester fatty acids amount in the luminal medium. Policosanol also significantly increased the free fatty acids amount in the luminal medium, while significantly decreased the free fatty acids amount in the cells. Policosanol slightly decreased the triglycerides fatty acids amount both in the luminal and lymphatic medium.

This study suggests two conclusions. First, very long chain fatty alcohols can be absorbed in intestine. Secondly, policosanol or policosanol metabolites after being

absorbed will modulate lipid metabolism or transport in intestine, such as fatty acid content in cholesteryl ester and triglycerides as well as free fatty acids, therefore, suggesting that policosanol may be oxidized and degraded into short-chain fatty acids after absorption in the intestine.

**Hypothesis 2:** Policosanol will modulate the lipid profile after being absorbed.

Further lipid profile evaluation demonstrated that policosanol significantly increased the total n-6 polyunsaturated fatty acids in the cholesterol ester in the lymphatic (basolateral) medium. The trend of the increasing contents include: linoleic acid (18:2n-6), gamma linoleic acid (18:3n-6), dinomo gamma linoleic acid (20:3n-6) and arachidonic acid (20:4n-6) with corresponding slightly decreasing content of lauric acid (C12) and myristic acid (C14). Policosanol significantly increased the proportion of behenic acid (C22) into the phospholipids in the lymphatic (basolateral) medium and corresponded to the slightly decreasing content of palmitic acid (C16).

Change in these lipid profiles in the lymphatic (basolateral) medium suggest that policosanol or the policosanol metabolites after absorption will modulate the lipid metabolism and profile in the Caco-2 cells to some degree. The amount modification is not apparently substantial.

## 5.2 DISCUSSION

Policosanol seems to be a safe (Pons et al., 1994; Castano et al., 2002; Mas et al., 1999) and low-cost phytochemical alternative to classic lipid-lowering agents, if it is proven to be efficacious. Several factors contribute to controversy about the effectiveness of policosanol. Firstly, policosanol is a mixture of very long chain aliphatic alcohols. The composition of policosanol varies depending on the source of material and preparation method used. Secondly, studies by varied groups report opposing results on the effectiveness of policosanol as a cholesterol-lowering agent and antioxidant. Thirdly, the absorption and metabolism of policosanol remains poorly understood. The present study indicates that the absorption of policosanol occurs in vitro.

## Policosanol composition and solubility

Policosanol composition used in all experiments is similar to that used by other groups for policosanol extracted from sugar cane wax. The major component is octacosanol, and accounts for 66% of the mixture. Policosanol has the highest solubility in chloroform following chloroform/methanol mixture (solubility 11.1 μg/ml and 8.3 μg/ml, respectively). Policosanol is not soluble in water. Phosphatidylcholine (PC) and bovine serum albumin (BSA) can deliver policosanol in the cell culture aqueous media. The PC and BSA carrier ability for policosanol is about 0.29 μg/mg and 0.75μg/mg, respectively. The binding ability of policosanol to BSA is more than two times that of PC, suggesting BSA has greater carrier ability to very long chain fatty alcohols.

## Cell viability

The cell viability study demonstrated that policosanol decreased the Caco-2 cell growth through days 2-4 of culture using PC or BSA as a carrier. Singh et al (1996) found that CaCo2 cells demonstrated an initial rapid phase of growth between Day 2 through days 7-9 of culture. The inhibitory effect is policosanol dose-dependent based on 48 hours incubation. The inhibitory effect starts from 1.14μg/ml when PC was used as a carrier. While the inhibitory effect starts from 0.05μg/ml when BSA was used, suggesting policosanol is likely effective even at a very low dose on the Caco-2 cell growth. The difference might result from the binding ability between PC and BSA, and the micelle composition. Noa et al. demonstrate that policosanol administered at 5 and 25 mg kg for 15 days to rabbits with cuffed arteries decreased the smooth muscle cell proliferation (Noa, et al. 1998). However, Roberto Menendez et al. found that 0.5, 5, and 50 μg/mL of policosanol have no effect on cultured fibroblast viability (Roberto Menendez, 2001). The discrepancy may contribute to the different characteristics of different cell lines.

In the present study, policosanol may inhibit Caco-2 cell growth by decreasing cell proliferation or inducing apoptosis. Several growth factors have been shown to play an important role in regulating Caco-2 cell growth, and abnormal expression of these growth factors has been related to cancer. Other investigators have described a possible important role of insulin-like growth factors (IGFs) and their binding proteins (IGFBPs)

in several types of cancer (Jones and Clemmons, 1995). IGFs are single-chain polypeptides with structural homology to proinsulin and are mitogens for a variety of mammalian cells (Jones and Clemmons, 1995). Besides endocrine effects, IGFs are autocrine and paracrine growth promoters of a variety of cells, and a family of IGF binding proteins (IGFBPs) modulates the biological actions of IGF-I and IGF-II (Kim et al 2000). All three components of the IGF system (IGFs, IGFBPs, and IGF receptors) may play an important role in the proliferation of colon cancers (Singh and Rubin, 1993).

Recently, Kim et al found that Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibited Caco-2 cells proliferation, and IGF-1I and IGFBP secretion revealed that low IGF-I1/IGFBP-6 ratios may have resulted in less free IGF-II and, consequently, the slower proliferation of Caco-2 cells treated with EPA or DHA (Kim et al., 2000). Similarly, Kim et al found that Conjugated linoleic acid (CLA ) decreased the Caco-2 cells growth, and suggested that the inhibitory effect is at least in part, be mediated by decreasing IGF-II secretion that is an autocrine growth stimulator of Caco-2 cells (Kim et al, 2002). The present study indicated that policosanol modulated the fatty acids amount and increased the n-6 polyunsaturated fatty acids (PUFA) of the cholesterol ester in the lymphatic (basolateral) medium. Further studies are warranted to confirm the underlying mechanism by which how policosanol inhibit the Caco-2 cells proliferation.

## Absorption and metabolism of policosanol

Morphological studies indicated Caco-2 cell monolayer is a suitable model to study the absorption and metabolism of very long chain fatty alcohols in intestine. The composition of policosanol and pure octacosanol did not undergo any change after 48 hours incubation in the Caco-2 cells monolayer. This suggests that very long chain fatty alcohols will not be elongated or degraded in the intestine and they have very similar absorption characteristics. It is about 86.0% after 48 hours incubation.

No other studies of cell cultures or clinical trials, or animal models have evaluated the effect of policosanol on cholesteryl ester fatty acids. Some clinical trials show that triglycerides were reduced to some extent in patients with type II hypercholesterolemia but mostly not significantly (Pons P et al, 1992; Aneiros E et al, 1995; Aneiros E et al, 1993). Results of free fatty acids amount show that policosanol treatment significantly

increased the amount of free fatty acids in the lymphatic (basolateral) medium, which is consistent with the reported by Menendez et al, who found that found that shortened saturated (myristic, palmitic and stearic) and unsaturated (oleic, palmitoleic) free fatty acids are formed in the plasma after oral dosing with policosanol to monkeys (Menendez et al, 2005). The present study also demonstrated that the free fatty acid amount is significantly decreased in the Caco-2 cells. Together, the present results suggests that policosanol or policosanol metabolites may modulate the transport or metabolism of free fatty acids as policosanol may be degraded into short-chain fatty acids. The present free fatty acids changes support the hypothesis from Kabir et al, who found that policosanol increases the amount of voluntary exercise in rats, and the underlying mechanism is possible that octacosanol increases the mobilization of free fatty acids from cells within muscle (Kabir et al, 1994).

Further evaluation of lipids profiles indicated that policosanol modulated the fatty acid profile of cholesterol ester in the lymphatic (basolateral) medium and phospholipids in the lymphatic (basolateral) medium, without change in the fatty acid profile in triglycerides and phospholipids of Caco-2 cell. The fatty acid profiles of cholesterol ester and phospholipids in the luminal (apical) medium were not influenced by policosanol.

It is possible that in the present study, the phospholipids fatty acids amount were not influenced by policosanol, because phosphatidylcholine (PC) was used as a carrier. 2mg/ml PC was added both in the control and policosanol treated cell culture, thus the high concentration may eliminate change in phospholipids composition or amount.

Further studies are warranted to evaluate the lipid profile change occurring in the free fatty acids fraction of Caco-2 cells, luminal (apical) and lymphatic (basolateral) medium.

## The potential effect of policosanol on cholesterol

HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA) and ACAT (acyl-CoA: cholesterol acyltransferase) control cholesterol synthesis and esterification, respectively, and the cholesterol ester present in the intestine is generated after cholesterol synthesis and a reaction catalyzed by ACAT (Marcil et al 2003). The present study demonstrated that policosanol influenced the cholesterol ester fatty acids amount in Caco-2 cells, apical

(luminal) and basolateral (lymphatic) medium. It is possible that policosanol or policosanol metabolites influence the HMG-CoA reductase and/or ACAT activity. But, further studies are required to evaluate the effect of policosanol on free cholesterol. Menendez et al also found that policosanol modulated HMG-CoA reductase activity in cultured fibroblasts when the cells were cultured in the lipoprotein-free medium (Menendez et al, 2001), but the investigators did not include any information on ACAT or cholesterol ester.

In summary, the present study indicated that policosanol will inhibit the Caco-2 cell proliferation. Policosanol absorption and metabolism studies in Caco-2 cell monolayers suggested that policosanol can be absorbed by small intestinal enterocytes. Policosanol or policosanol metabolites will modulate lipid metabolism or/and transport in the Caco-2 cells, after being absorbed. However, future studies are required for a more complete understanding of the specific mechanisms and inhibitory effects of policosanol on Caco-2 cells and on the modulation of lipid metabolism, and free cholesterol in this cell line.

# Chapter 6

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