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Policosanol Inhibits Cholesterol Synthesis in Hepatoma Cells by Activation of AMP-kinase

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ABSTRACT

Policosanol is a mixture of long-chain primary alcohols that has been shown to decrease serum cholesterol in animals and in man. The hypocholesterolemic effect appears to result from a decrease in cholesterol synthesis by suppression of HMG-CoA reductase activity, but the mechanism of this suppression and the active components of policosanol have not been established. In the present study we investigated the ability of policosanol and its principal components to inhibit cholesterol synthesis in cultured rat hepatoma cells. Maximal inhibition by policosanol yielded a 30% decrease in ^{14}C -acetate incorporation without evidence of cellular toxicity. Octacosanol (C_{28} , the major constituent of policosanol), heptacosanol (C_{27}), and hexacosanol (C_{26}) yielded smaller and statistically insignificant decreases in cholesterol synthesis, whereas triacontanol (1-hydroxytriacontane, C_{30}) replicated the inhibition obtained with policosanol. At pharmacologic concentrations ($<5\text{ }\mu\text{g/ml}$) policosanol and triacontanol decreased ^{14}C -acetate incorporation into cholesterol without affecting the incorporation of ^{14}C -mevalonate, indicating that these compounds act at or above HMG-CoA reductase. Policosanol and triacontanol did not directly inhibit HMG-CoA reductase, and incubation of these compounds with hepatoma cells did not affect reductase enzyme levels. However, reductase activity was decreased by up to 55% in lysates prepared from these cells, suggesting that HMG-CoA reductase activity was down-regulated by policosanol treatment. Consistent with this hypothesis, a three-fold increase in AMP-kinase phosphorylation was noted in policosanol-treated cells. As AMP-kinase is activated by phosphorylation and is well established to suppress HMG-CoA reductase

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activity, these results suggest that policosanol or a metabolite decreases HMG-CoA reductase activity by activating AMP-kinase.

Introduction

Policosanol, a mixture of very long-chain alcohols isolated from sugar cane, at doses of 10-20 mg/day has been shown to lower total and LDL cholesterol by up to 30%, equivalent to low-dose statin therapy (Gouni-Berthold and Berthold, 2002). In both short-term (≤ 12 wk) and long-term (up to 2 yr) randomized, placebo-controlled, double-blind studies policosanol lowered LDL-cholesterol in normocholesterolemic patients by an average of 33%, and in hypercholesterolemic patients by 24% (reviewed in Gouni-Berthold and Berthold, 2002; Varady et al., 2003). In normocholesterolemic patients policosanol caused a small and generally insignificant increase in HDL-cholesterol, whereas in seven clinical studies of dyslipidemic patients HDL-cholesterol was increased by an average of 17%. Policosanol is also effective in rabbits and monkeys, where it lowers blood cholesterol and reduces the development of atherosclerotic plaques (Arruzazabala et al., 1994; Rodriguez-Echenique et al., 1994; Menendez et al., 1997), but was found not to be effective in hamsters (Wang et al., 2003).

The major components of policosanol are the primary alcohols octacosanol (C_{28} , ~60%), triacontanol (C_{30} , 12-14%), and hexacosanol (C_{26} , 6-12%), with lesser amounts of other alcohols with chain lengths of 24 to 34 carbons. The product has no evident toxicity and is available over-the-counter in many outlets. The active component(s) have not been established, but it has been shown that very long chain alcohols can undergo oxidation to fatty acids with subsequent peroxisomal β -oxidation, which also yields chain-shortened metabolites (Singh et al., 1987). D-003, a mixture of very long chain saturated fatty acids also purified from sugar cane, similarly lowers LDL and total cholesterol in normocholesterolemic patients (Castano et al., 2002) and in

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normocholesterolemic and casein-induced hypercholesterolemic rabbits, and a more rapid onset of effects suggests that oxidation of policosanols to very long chain fatty acids may be necessary for their hypocholesterolemic actions (Menendez et al., 2004).

Several studies have demonstrated that policosanol inhibits cholesterol synthesis in laboratory animals and cultured cells, and it is thought that this is the principal mechanism by which it lowers blood cholesterol levels. Policosanol reduced the incorporation of tritiated water into sterols in hypercholesterolemic rabbits (Menendez et al., 1997) and decreased ^{14}C -acetate incorporation into cholesterol in human fibroblasts (Menendez et al., 2001a). In the latter study policosanol did not affect the incorporation of ^{14}C -mevalonate into cholesterol, indicating that policosanol was acting at or above mevalonate synthesis. However, policosanol did not inhibit HMG-CoA reductase (mevalonate synthase) when added to cell lysates, arguing against a direct interaction with this enzyme. The ability of policosanol to prevent the up-regulation of HMG-CoA reductase activity in these cells in response to lipid-depleted media suggested that policosanol suppresses HMG-CoA reductase synthesis or enhances enzyme degradation. Similar results were obtained with D-003 (Menendez et al., 2001b), although neither study measured HMG-CoA reductase protein levels. The present studies were undertaken to further explore the mechanism by which policosanol inhibits cholesterol synthesis and to identify the active component(s) of this natural product.

Materials and Methods

Chemicals. Policosanol 10 mg tablets were manufactured by Source Naturals, Inc. (Scotts Valley, CA), and purchased from a local health-foods store. Hexacosanol, heptacosanol, octacosanol, triacontanol (1-hydroxytriacontane), mevalonolactone, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADH, lactate dehydrogenase, pyruvate, Triton X100, ketoconazole, squalene, lanosterol, and mevalonolactone were purchased from Sigma Chemical Co. (St Louis, MO). Dulbecco's modified media (DMEM), penicillin-streptomycin-glutamine (PSG, 100X), fetal bovine serum (FBS), lipoprotein-depleted serum, and trypsin were purchased from Invitrogen (GIBCO, Carlsbad, CA). Terbinafine was from TCI America, Inc. (Portland, OR). ^{14}C -Acetate, sodium salt (56 mCi/mmol) and ^{14}C -mevalonate, DBED salt (65 mCi/mmol) were purchased from Amersham, Inc. (Piscataway, NJ); ^{14}C -HMG-CoA (55 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Preparation and Analysis of Policosanol. Two 10-mg policosanol tablets were dissolved in 10 ml of absolute ethanol with a mortar and pestle. The insoluble excipients were removed by low-speed centrifugation and the supernatant was aliquoted and stored at -20°C , and added directly to media or buffer upon use. For the mass-spectrometric analysis the tablets were dissolved in methylene chloride, fractionated by gas chromatography (Trace) and quantified by ion-trap mass spectrometry (ThermoFinnigan PolarisQ) at the University of Kentucky Mass Spectrometry Facility.

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Cytotoxicity Assays. McARH7777 rat hepatoma cells (ATCC, Rockville, MD) were grown in DMEM supplemented with 10% FBS and 1.0% PSG in 6-well plates at 37°C under a humidified atmosphere of 5% CO₂. The cells were used between passages 1 and 40. After 48 h the media was replaced with fresh media with the addition of policosanol (2 mg/ml in ethanol) or a long-chain alcohol (1mg/ml in ethanol) and incubation was continued for 3 h. Control cells received an equal volume of ethanol. The cells were detached with trypsin, suspended in DMEM containing 0.2% trypan blue, and counted in a hemocytometer. Leakage of lactate dehydrogenase from cells was determined by measuring NADH oxidation from added pyruvate spectrophotometrically at 340 nm and compared to total lactate dehydrogenase activity from cells lysed with 0.1% Triton X100.

Determination of Sterol Synthesis. Hepatoma cells were cultured for 48 h in 6-well plates, at which time the media was replaced and appropriate concentrations of the test substances (policosanol, 2 mg/ml in ethanol, or long-chain alcohol, 1 mg/ml in ethanol) were added along with 1 μ Ci of ¹⁴C-acetate or ¹⁴C-mevalonate. Incubations were carried out for 3 h, after which time the cells were washed twice with phosphate-buffered saline, harvested by trypsinization or scraping, resuspended in 20 mM Tris buffer, pH 7.4, containing 0.1% Triton X100, and lysed by sonication (Sonic Dismembrator, Fisher Scientific) at medium setting on ice with ten 8-sec pulses, separated by 30 sec each. Lipids were extracted into 5 ml of chloroform:methanol (2:1), the solvent was removed by evaporative centrifugation, and the lipids were resuspended in 50 μ l of chloroform/methanol and spotted onto silica thin layer plates (Whatman). Chromatography was carried out in petroleum ether:ethyl ether:acetic acid

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(60:40:1). Cholesterol was identified by co-chromatography of an authentic standard visualized by iodine vapor staining and quantified by electronic autoradiography (Packard Instant Imager). Further confirmation of identity was obtained by scraping the corresponding region of nonradiolabeled samples into chloroform/methanol (2:1), derivatizing the samples with trimethylsilane, and submitting them to mass spectrometric analysis as described above for policosanol.

Determination of Squalene and Lanosterol Synthesis. For the determination of squalene and lanosterol synthesis, cells were incubated as described above for cholesterol synthesis with the inclusion of 60 μ M terbinafine (100x in ethanol), an inhibitor of squalene monooxygenase (for the determination of squalene), or 10 μ M ketoconazole (100x in methanol), an inhibitor of lanosterol demethylase (for the determination of lanosterol). Lipids were saponified by addition of 0.5 ml of 10% methanolic potassium hydroxide and incubated at 80°C for 1 h. For the determination of squalene the neutral lipids were extracted into 5 ml of petroleum ether, the solvent was removed by centrifugal evaporation, and the samples were resuspended in 50 μ l of petroleum ether and resolved by silica thin-layer chromatography in 5% ethyl acetate in hexane. Lanosterol was determined as described for cholesterol. Authentic standards for squalene and lanosterol were visualized by iodine-vapor staining. Further confirmation of these products was obtained by scraping the corresponding region of nonradiolabeled samples into chloroform/methanol (2:1) and submitting them to mass spectrometric analysis as described above for policosanol.

Determination of HMG-CoA Reductase Activity in Microsomes. The microsomal fraction (100,000xg pellet resuspended at ~15 mg protein/ml) was prepared by standard

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procedures from the livers of untreated male Harlan Sprague-Dawley rats (~200 g). HMG-CoA reductase activity was determined by the procedure of Brown and Goldstein (1974) as follows: Microsomes (100 µg) were incubated at 37°C in a final volume of 200 µl containing 0.1 M potassium phosphate buffer, pH 7.5, 20 mM glucose 6-phosphate, 2.5 mM NADP⁺, 1 unit of glucose 6-phosphate dehydrogenase, 5 mM dithiothreitol, and 0.2 µCi ¹⁴C-HMG-CoA. The reaction was stopped after 3 h by the addition of 25 µl of 6M HCl. Mevalonate was converted to the lactone by standing at 37°C for 30 min, extracted into 5 ml of ethyl acetate, and brought to dryness by evaporative centrifugation. The sample was redissolved in 50 µl of ethyl acetate and fractionated by silica thin-layer chromatography with benzene:acetone (1:1). Mevalonolactone was identified by co-migration with authentic mevalonolactone visualized by iodine vapor staining and quantified by electronic autoradiography.

Determination of HMG-CoA Reductase Activity in Cell Culture. Policosanol or a long-chain alcohol was added as indicated in fresh media to 48-h hepatoma cell cultures and incubated for 3 h. Cells were lysed by sonication and HMG-CoA reductase activity was determined with 100 µg of total cell lysate protein as described above.

Immunoblot Analysis of HMG-CoA Reductase Expression in Cell Culture. Policosanol or a long-chain alcohol was added as indicated in fresh media to 48-h hepatoma cell cultures and incubated for up to 12 h. Cells were lysed by sonication and 50 µg of total cell protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis. The proteins were electroblotted to nitrocellulose, the membrane was blocked with 0.5% Tween-20 and 5% defatted milk, and then incubated with rabbit antibody to HMG-CoA reductase (1:2000; Upstate USA, Inc.). The

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immunoblot was developed with a secondary antibody conjugated to alkaline phosphatase and visualized by bromochloroindolyl phosphate/nitro-blue tetrazolium staining.

Immunoquantitation of AMP-kinase. Hepatoma cells (rat McARH7777 or human HepG2) were cultured in lipoprotein-deficient media overnight, after which 20 μ g of policosanol was added and cells were incubated for 1 or 3 h. Cells were washed once with phosphate-buffered saline, scraped from the plates, pelleted by low-speed centrifugation, and lysed by two cycles of freeze-thawing in 0.25 M Tris buffer, pH 7.5, containing protease and phosphatase inhibitor cocktails (Sigma). For the metformin comparison studies metformin (0.5 mM to 2 mM, Sigma) or policosanol (10 or 20 μ g/ml) was added and incubation continued for 3-6 h, following the protocol of Zang et al. (2004). The lysates were fractionated by centrifugation (20,000 x g, 15 min, at 4°C) and the supernatant was collected and stored at -80°C. Twenty-five μ g of protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis and electroblotted to nitrocellulose. The membrane was blocked with 0.05% Tween-20 and 5% defatted milk, and then incubated in this same buffer with rabbit antibody to total AMP-kinase (Anti-AMPK α -pan, 1:2000; Upstate USA, Inc.) or to phosphorylated AMP-kinase (Anti-phospho-AMPK α , 1:1000; Upstate USA, Inc.). The immunoblot was developed with a secondary antibody conjugated to horseradish peroxidase and visualized and quantified by chemiluminescence (Supersignal West Pico Chemiluminescent Substrate, Pierce) on a Kodak Image Station.

Results

Policosanol is a commercial mixture of very long chain alcohols composed primarily of octacosanol (~60%), triacontanol (~13%), and hexacosanol (~6%). To evaluate the composition of our commercial preparation we extracted the alcohols into methylene chloride and subjected the extract to gas chromatography. Five major peaks were isolated and identified by mass spectrometry (Fig. 1). Peaks A and B correspond to short-chain carboxylic acid excipients used to prepare the tablets; peaks C, D, and E correspond to hexacosanol (16%), octacosanol (60%), and triacontanol (19%). Minor components (not identified) comprise approximately 5% of the material eluting between 14 and 30 min, and may include C₂₇ (heptacosanol) and C₂₉ (nonacosanol) alcohols.

The addition of policosanol to McARH7777 rat hepatoma cells resulted in a dose-dependent decrease in cholesterol labeling from ¹⁴C-acetate (Fig. 2). Maximal inhibition (~30%) was obtained at 25 µg/ml and higher concentrations, up to 50 µg/ml, did not yield greater inhibition. Policosanol at concentrations up to 50 µg/ml did not affect cell viability as assessed by trypan blue exclusion or the release of lactate dehydrogenase into the media. Triacontanol was similarly effective, whereas hexacosanol yielded a lesser (~20%) and statistically insignificant decrease in cholesterol labeling. Heptacosanol, which was not present in our preparation, decreased cholesterol synthesis by less than 10%, and octacosanol, the most abundant alcohol in policosanol, yielded only a 15% decrease in cholesterol labeling at 25 µg/ml. The combination of triacontanol with hexacosanol and octacosanol was slightly more inhibitory than triacontanol alone at the same concentration (5 µg/ml). None of the alcohols proved toxic to the cells at concentrations up to 50 µg/ml.

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The possibility that policosanol was decreasing ^{14}C -acetate uptake was examined by measuring total ^{14}C incorporation into cells after 30-min incubations with policosanol and the radiolabel. ^{14}C -acetate uptake was decreased substantially at concentrations of 5 $\mu\text{g/ml}$ and above (Fig. 3A), and cholesterol labeling was similarly decreased at these concentrations. In contrast, acetate uptake was not affected by policosanol concentrations below 5 $\mu\text{g/ml}$, whereas cholesterol synthesis was decreased by ~20% between 0.5 and 5 $\mu\text{g/ml}$ (Fig. 3B). Triacantanol similarly decreased acetate uptake (data not shown). In contrast to the effect of policosanol on acetate uptake, policosanol did not affect mevalonate uptake into hepatoma cells (Fig. 3C). At concentrations above 5 $\mu\text{g/ml}$ policosanol decreased the incorporation of ^{14}C -mevalonate into cholesterol by as much as 50%, whereas at concentrations below 5 $\mu\text{g/ml}$ policosanol had no effect on cholesterol synthesis from mevalonate (Fig. 3D). Triacantanol had a similar effect on cholesterol synthesis, decreasing the incorporation of ^{14}C -acetate, but not ^{14}C -mevalonate, into cholesterol by ~20% at 5 $\mu\text{g/ml}$ and decreasing the incorporation of both acetate and mevalonate by up to 35% at 25 $\mu\text{g/ml}$ (data not shown). Taken together, these results suggest that policosanol inhibits cholesterol synthesis at or prior to mevalonate synthesis at concentrations below 5 $\mu\text{g/ml}$.

Although the inhibition of cholesterol synthesis at low concentrations of policosanol (<5 $\mu\text{g/ml}$) appeared to take place at or above mevalonate synthesis, higher concentrations of policosanol appeared to act downstream of mevalonate synthesis. To try to identify this site of inhibition, the labeling of lanosterol, a mid-point intermediate in the cholesterol biosynthetic pathway, was determined in the presence of various concentrations of policosanol (Fig. 4A). As with cholesterol synthesis, lanosterol

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synthesis from ^{14}C -acetate was decreased at very low concentrations of policosanol, consistent with it acting early in the biosynthetic pathway. When ^{14}C -mevalonate was supplied as the precursor, lanosterol synthesis was not impaired until the policosanol concentration reached 10 $\mu\text{g/ml}$, as seen with cholesterol synthesis from mevalonate. A similar result was obtained when squalene synthesis was measured in the presence of various concentrations of triacontanol (Fig. 4B). The decrease in labeling of squalene and lanosterol from mevalonate suggests that higher concentrations of policosanol act somewhere between HMG-CoA reductase and squalene synthase to block synthesis. Alternatively, the greater inhibition of HMG-CoA reductase at higher concentrations of policosanol or triacontanol may lead to a decrease in mevalonate synthesis that cannot be overcome by the exogenously supplied ^{14}C -mevalonate.

To determine if policosanol and triacontanol directly inhibit HMG-CoA reductase, these compounds were added to rat liver microsomes and the conversion of ^{14}C -HMG-CoA to mevalonate was measured. As shown in Fig. 5A, neither compound affected HMG-CoA reductase activity, indicating that they did not act as direct inhibitors of this enzyme. However, when hepatoma cells were incubated for 3 h with these compounds and HMG-CoA reductase activity was measured in the cell lysates both policosanol and triacontanol reduced enzyme activity by as much as 55% (Fig. 5B). These results suggest that either policosanol requires metabolism to form the active inhibitor, or that policosanol reduces the expression of HMG-CoA reductase by decreasing transcription and translation or by enhancing degradation of the enzyme. To determine if policosanol alters HMG-CoA reductase expression, cells were incubated with policosanol or triacontanol for 3 h and enzyme levels were evaluated by immunodetection. As shown

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in Fig. 5C, policosanol and triacontanol did not decrease the amount of HMG-CoA reductase protein over the 3-h period of the experiment, despite a marked decrease in the activity of this enzyme.

Since HMG-CoA reductase protein levels were not changed by policosanol treatment, we considered the possibility that policosanol inactivates HMG-CoA reductase by promoting its phosphorylation by one of three protein kinases shown to inactivate HMG-CoA reductase (Beg et al., 1987a). As shown in Fig. 6A, policosanol increased the amount of phosphorylated AMP-kinase in cells by over 3-fold after 3 h of treatment. Similar results were obtained with the human hepatoma cell line, HepG2 (Fig. 6B), indicating that this is not a species-specific effect; metformin, which is known to promote the phosphorylation of AMP-kinase (Zhou et al., 2001), was similarly effective. AMP-kinase is well established to be a regulator of HMG-CoA reductase activity in response to changes in cellular energy levels, and is activated by phosphorylation by one or more upstream kinases, including LKB1 (Hawley et al., 2003; Shaw et al., 2004). These results suggest that policosanol decreases HMG-CoA reductase activity by activating AMP-kinase.

Discussion

Policosanol is an effective inhibitor of cholesterolgenesis in hepatoma cells at concentrations that are readily obtainable in vivo: a single 20-mg oral dose in a 70-kg man would yield a total body water concentration of up to 0.5 $\mu\text{g/ml}$, and in the present studies this concentration of policosanol reduced cholesterol synthesis by ~15% (Fig. 3B). Moreover, as oral dosing is likely to result in significant hepatic exposure, the effective concentration therein is likely to be substantially higher; indeed, Kabir and Kimura (1995) reported that radiolabeled octacosanol is predominantly located in the liver after oral dosing. Policosanol reduces LDL-cholesterol by 20-30% and increases hepatic LDL-receptor expression (Menendez et al., 1997), both of which are consistent with down-regulation of hepatic cholesterolgenesis. Numerous clinical studies have documented the ability of policosanol to reduce serum cholesterol in both normocholesterolemic and hypercholesterolemic patients (reviewed in Gouni-Berthold and Berthold, 2002; Varady et al., 2003; see also Castano et al., 2005; Wright et al., 2005), although a very recent clinical study was unable to find a significant cholesterol-lowering effect (Berthold et al., 2006).

Despite the evidence that policosanol reduces cholesterol synthesis, the active principal(s) and the mechanism have not been established. Our studies reveal that triacontanol, a C_{30} primary alcohol that makes up 19% of the very long-chain alcohols present in our preparation, appears largely responsible for the inhibition of cholesterol synthesis in hepatoma cells. Octacosanol, the principal component of policosanol, afforded only a small reduction in cholesterol synthesis even at suprapharmacologic concentrations. At pharmacologic concentrations ($<5 \mu\text{g/ml}$, 11 pM) triacontanol

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inhibited squalene and cholesterol synthesis from ^{14}C -acetate but not from ^{14}C -mevalonate, indicating a site of action at or above HMG-CoA reductase. Triacontanol effectively decreased HMG-CoA reductase activity in hepatoma cells but did not directly inhibit this enzyme when added to rat liver microsomes, and neither triacontanol nor policosanol affected HMG-CoA reductase enzyme levels in these short-term assays. These findings suggested that these very long chain alcohols were either being metabolized to an active enzyme inhibitor, or were acting via an intracellular regulatory pathway.

HMG-CoA reductase has been shown to be subject to regulation by reversible phosphorylation by several protein kinases, including AMP-activated kinase (Ferrer et al., 1985), a protein kinase C (Beg et al., 1985), and a calmodulin-dependent protein kinase (Beg et al., 1987b). AMP-kinase, which also inactivates acetyl-CoA carboxylase (Carling et al., 1987), appears to be the major regulator of HMG-CoA reductase phosphorylation, and its co-regulation of acetyl-CoA carboxylase suggests coordinate regulation of cholesterol and fatty acid biosynthesis. AMP-kinase is activated by 5'-adenosine monophosphate, which increases in cells during ATP depletion consequent to various stresses (hypoxia, ischemia, glucose depletion), as well as to excessive energy demands (Hardie, 2003). Activation of AMP-kinase requires phosphorylation of the catalytic unit by one or more upstream kinases, including LKB1 (Kahn et al., 2005), and indeed, long-chain fatty acids *per se* appear to activate AMP-kinase via a phosphorylation mechanism (Clark et al., 2004). Our findings demonstrate that policosanol promotes the phosphorylation of AMP-kinase in hepatoma cells, suggesting that this is the likely mechanism by which HMG-CoA reductase activity is reduced in

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treated cells. It remains unclear if the very long-chain alcohols in policosanols must first undergo oxidative metabolism via the fatty alcohol cycle (Rizzo et al., 1987) to the corresponding fatty acids or subsequent peroxisomal β -oxidation. Pharmacokinetic studies on octacosanol metabolism have indicated that this very long chain alcohol can undergo oxidation to CO_2 *in vivo*, presumably via this pathway (Kabir and Kimura, 1993).

It is not clear why triacontanol was more effective than the other long-chain alcohols we tested, as all should yield very long-chain fatty acids, and all should undergo peroxisomal β -oxidation. Triacontanol may represent the minimal effective length (C-30), as the other alcohols we tested were 2-4 carbon atoms shorter. While policosanols are composed primarily of C-30 and shorter aliphatic alcohols (Menendez et al., 1997, and present studies), D-003, a mixture of very long-chain fatty acids that similarly inhibits cholesterol synthesis in cultured cells (Menendez et al., 2001b), contains a significant proportion of fatty acids greater than 30 carbons in length. This may contribute to its suggested greater effectiveness in lowering blood cholesterol levels (Menendez et al., 2004).

Suprapharmacologic concentrations of policosanols impaired the uptake of ^{14}C -acetate into these cells. Thus, at higher policanol concentrations the decrease in cholesterol labeling from ^{14}C -acetate could not exclude decreased uptake of label as a cause. However, we suspect that the decrease in uptake of ^{14}C -acetate may actually reflect decreased incorporation into fatty acids in the presence of high levels of policosanols, as fatty acid synthesis in untreated cells represents a significant “sink” for radiolabeled acetate. If policanol acts via the AMP-kinase pathway it would be

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expected that fatty acid synthesis would also be suppressed, as AMP-kinase regulates acetyl CoA carboxylase, the first and regulatory step in fatty acid synthesis (Hardie and Pan, 2002). Mevalonate uptake was not affected by policosanol, consistent with its more limited role in cellular biochemistry; however, higher concentrations of policosanol decreased mevalonate incorporation into cholesterol, suggesting the possibility of additional sites of inhibition downstream of HMG-CoA reductase. Thin-layer chromatographic analysis did not reveal the accumulation of sterol precursors or intermediates that would indicate additional sites of inhibition; the decrease in mevalonate incorporation at higher policosanol levels may reflect decreased mevalonate synthesis by HMG-CoA reductase, thereby limiting overall flux through the pathway in the presence of sub-saturating levels of radiolabeled mevalonate. We conclude that suppression of HMG-CoA reductase activity is the principal mechanism by which policosanol decreases cholesterol synthesis.

The observation that policosanol activates AMP-kinase is reminiscent of the mechanism of metformin, a drug widely used to treat type II diabetes. Metformin (Glucophage) acts through AMP-kinase to reduce blood glucose levels, enhance glucose uptake into skeletal muscle, decrease circulating lipids, and inhibit hepatic gluconeogenesis (Zhou et al., 2001). Metformin requires the presence of LKB1, the kinase that activates AMP-kinase, for its anti-diabetic effects (Shaw et al., 2005). A comparison of policosanol to metformin in their ability to stimulate AMP-kinase phosphorylation in the human hepatoma cell line, HepG2, revealed that policosanol at 25 $\mu\text{g/ml}$ was as efficacious as metformin, while being considerably more potent: 20 $\mu\text{g/ml}$ ($\sim 8 \mu\text{M}$ triacontanol) was equivalent to 265 $\mu\text{g/ml}$ (1.6 mM) metformin in this *in*

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vitro assay (Fig. 6B and data not shown). These results, taken in total, suggest that an evaluation of the ability of policosanol to moderate blood glucose levels in type II diabetes may be warranted.

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Footnotes

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Figure Legends

Fig. 1. Gas chromatogram of policosanol. Peaks A, B, C, D, and E were analyzed by mass spectrometry and shown to correspond to **A**, C₁₆ carboxylic (palmitic) acid; **B**, C₁₈ carboxylic (stearic) acid; **C**, C₂₆ alcohol (hexacosanol); **D**, C₂₈ alcohol (octacosanol); and **E**, C₃₀ alcohol (triacontanol).

Fig. 2. Inhibition of cholesterol synthesis by policosanol and its components. Cholesterol synthesis from ¹⁴C-acetate was determined in 3-h cultures in the presence of policosanol or its principal components. Values represent the mean and standard error of 2-5 experiments carried out in duplicate. Asterisk (*) indicates statistical significance with respect to untreated cells as determined by one-way ANOVA with Tukey's post-hoc test.

Fig. 3. Sterol precursor uptake and incorporation into cholesterol. ¹⁴C-Acetate or ¹⁴C-mevalonate content or incorporation was measured in hepatoma cells at various concentrations of policosanol. **A**, ¹⁴C-Acetate uptake and incorporation; **B** expands the 0-5 µg concentration segment of graph **A**. **C**, ¹⁴C-Mevalonate uptake and incorporation; **D** expands the 0-5 µg concentration segment of graph **C**. Each point represents the mean and standard error of 1-3 experiments carried out in duplicate.

Fig. 4. Inhibition of lanosterol and squalene synthesis by policosanol and triacontanol. The labeling of lanosterol (**A**) and squalene (**B**) from ¹⁴C-acetate and ¹⁴C-mevalonate was measured in hepatoma cells incubated in the presence of various

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concentrations of policosanol or triacontanol. Each value represents the mean and standard error of 2-4 experiments carried out in duplicate. Representative thin-layer chromatograms are shown to the right of each graph; lane 1 shows the synthesis of lanosterol (*La*) or squalene (*Sq*) in the absence of policosanol or triacontanol, and lane 2 shows the decrease in synthesis in the presence of these very long chain alcohols.

Fig. 5. Effect of policosanol and triacontanol on HMG-CoA reductase activity. HMG-CoA reductase activity was measured in microsomes prepared from rat liver to which policosanol or triacontanol was added as indicated (**A**) or in lysates prepared from hepatoma cells incubated with the indicated concentrations of policosanol or triacontanol (**B**). Each value represents the mean and standard error of two experiments carried out in duplicate. **C**, Immunoblot of HMG-CoA reductase. Hepatoma cells were incubated with the indicated concentrations of policosanol or triacontanol for 3 h, after which time cell lysates were prepared, fractionated by electrophoresis and transferred to nitrocellulose for immunodetection with an antibody specific for HMG-CoA reductase.

Fig. 6. Immunoblot of total and phosphorylated AMP-kinase. **A**, Hepatoma cells were untreated (lanes 1 and 3) or incubated with 20 μ g/ml of policosanol for 1 h (lanes 2 and 5) or 3 h (lanes 3 and 6), after which cell lysates were prepared and a low-speed supernatant was fractionated by electrophoresis and transferred to nitrocellulose for immunodetection with an antibody specific for total (α -AMPK, lanes

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1-3) or phosphorylated (α -P-AMPK, lanes 4-6) AMP-kinase. Lane 7 contains molecular mass markers (60 and 80 kDa); lane 8 contains pure AMP-kinase, detected with α -AMPK antibody. The relative intensity values are indicated under each lane, with untreated (Co) set at 1.0. **B**, HepG2 hepatoma cells were incubated for 6 h with policosanol or metformin at the indicated concentrations and phosphorylated AMP-kinase in cell lysate supernatants was quantified as described above. The relative intensity values are indicated under each lane, with untreated (Co) set at 1.0.

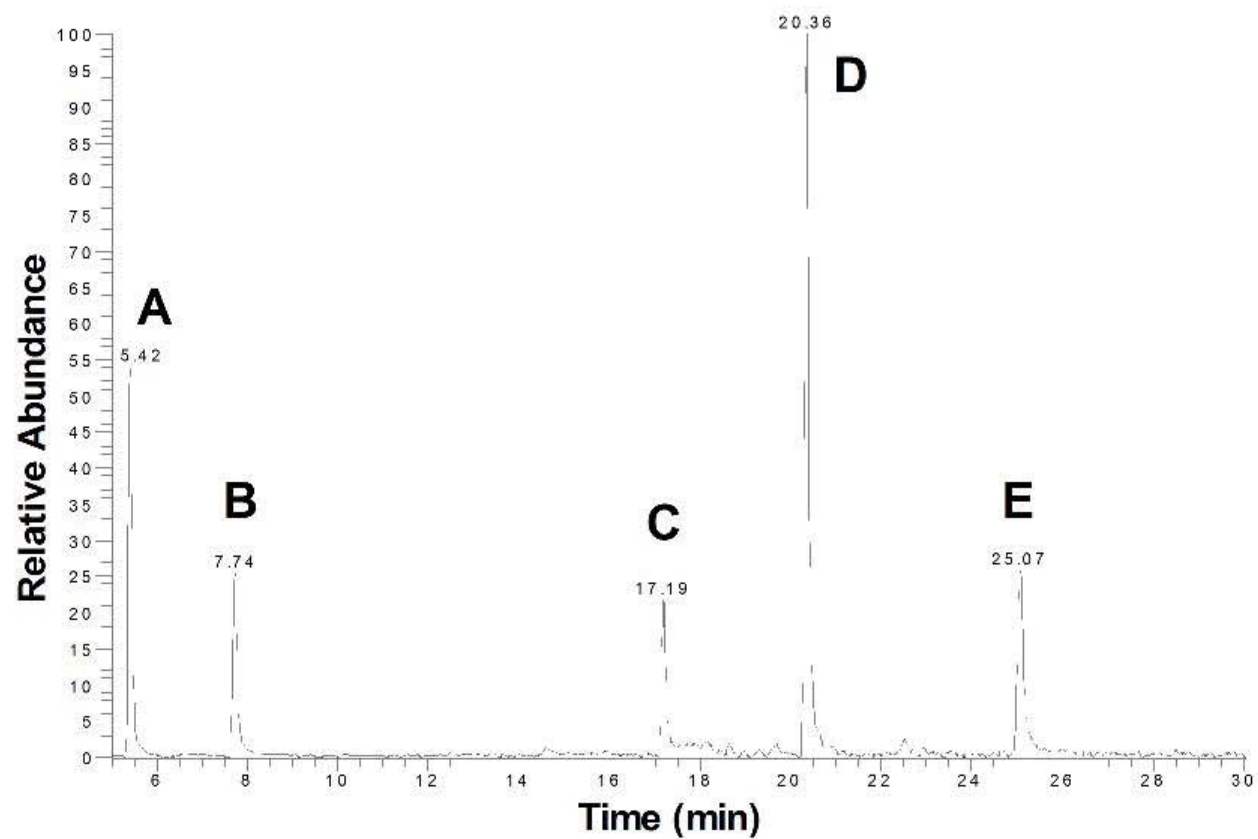
Fig. 1

Fig. 2

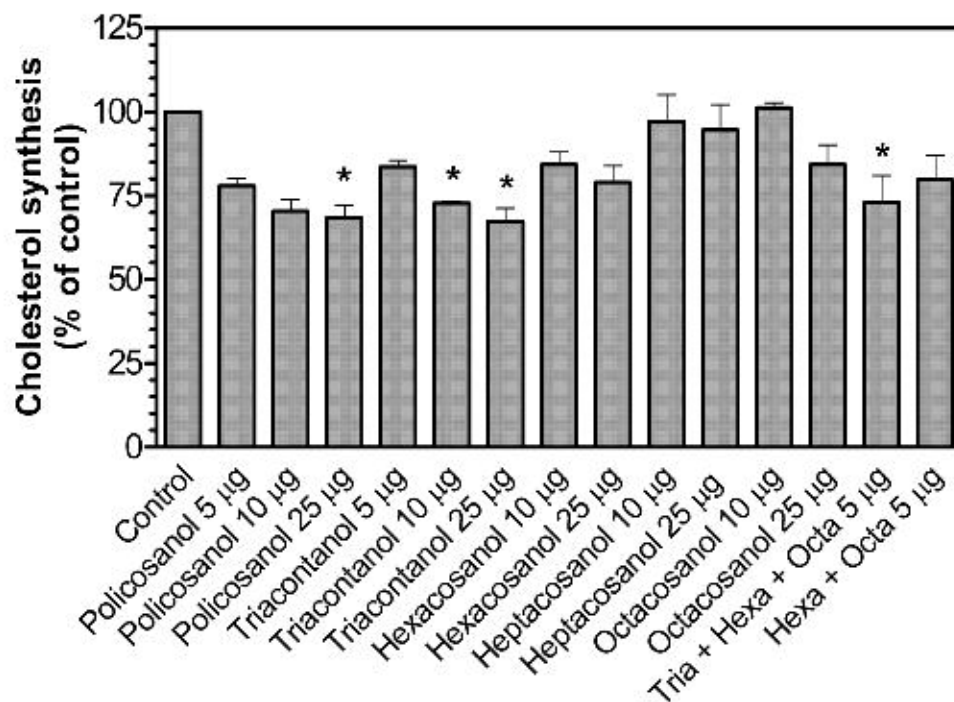


Fig. 3

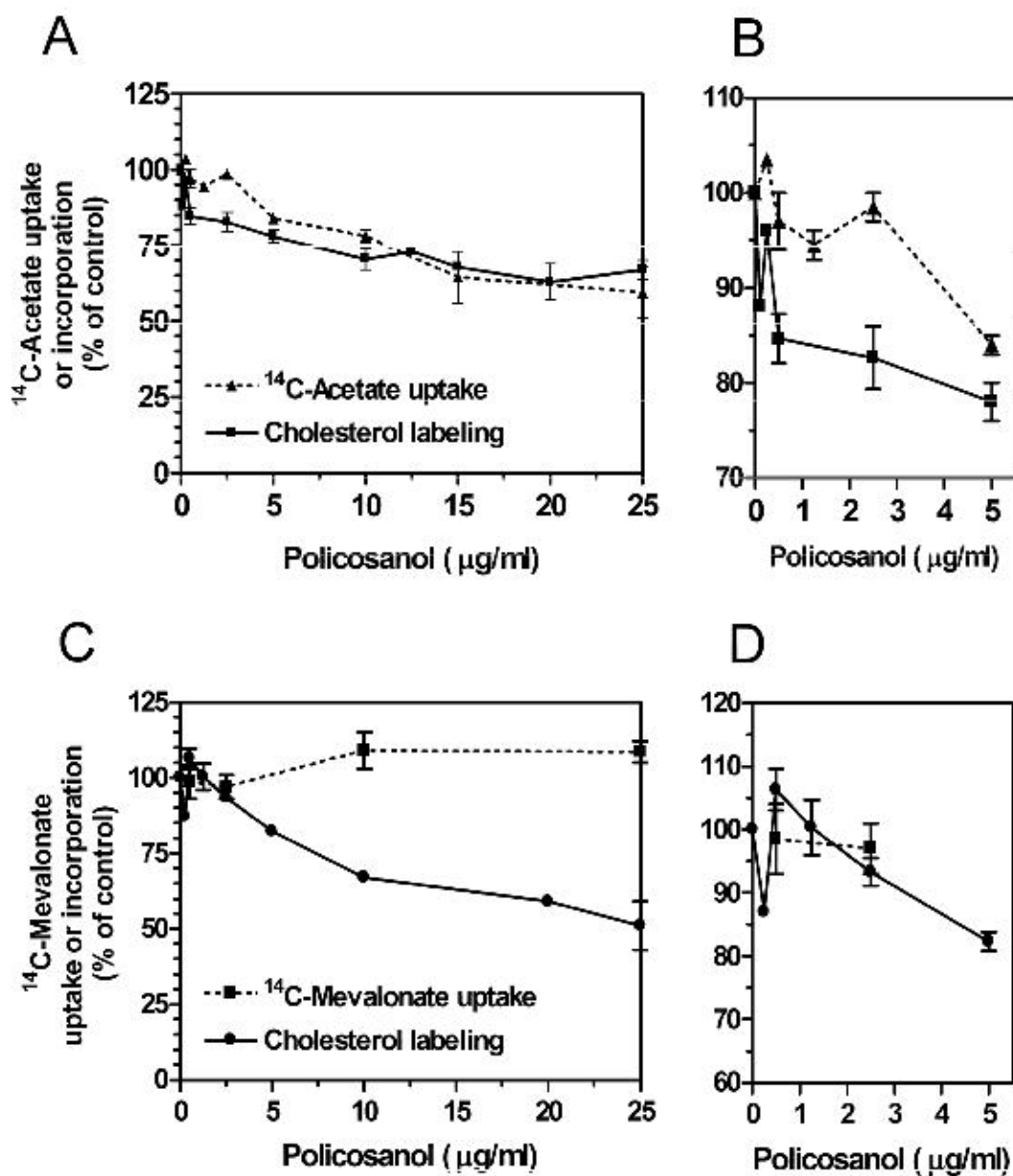


Fig. 4

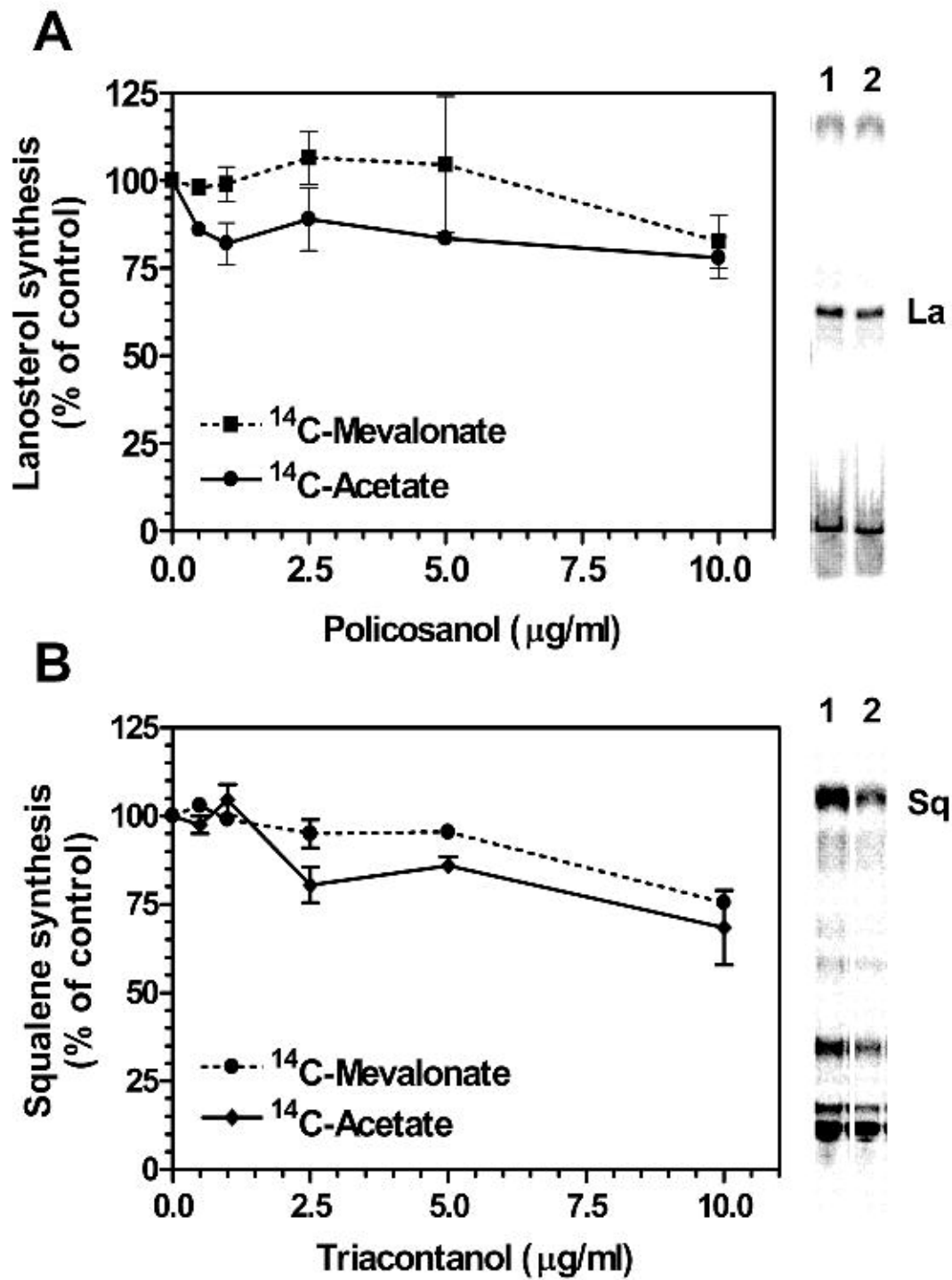


Fig. 5

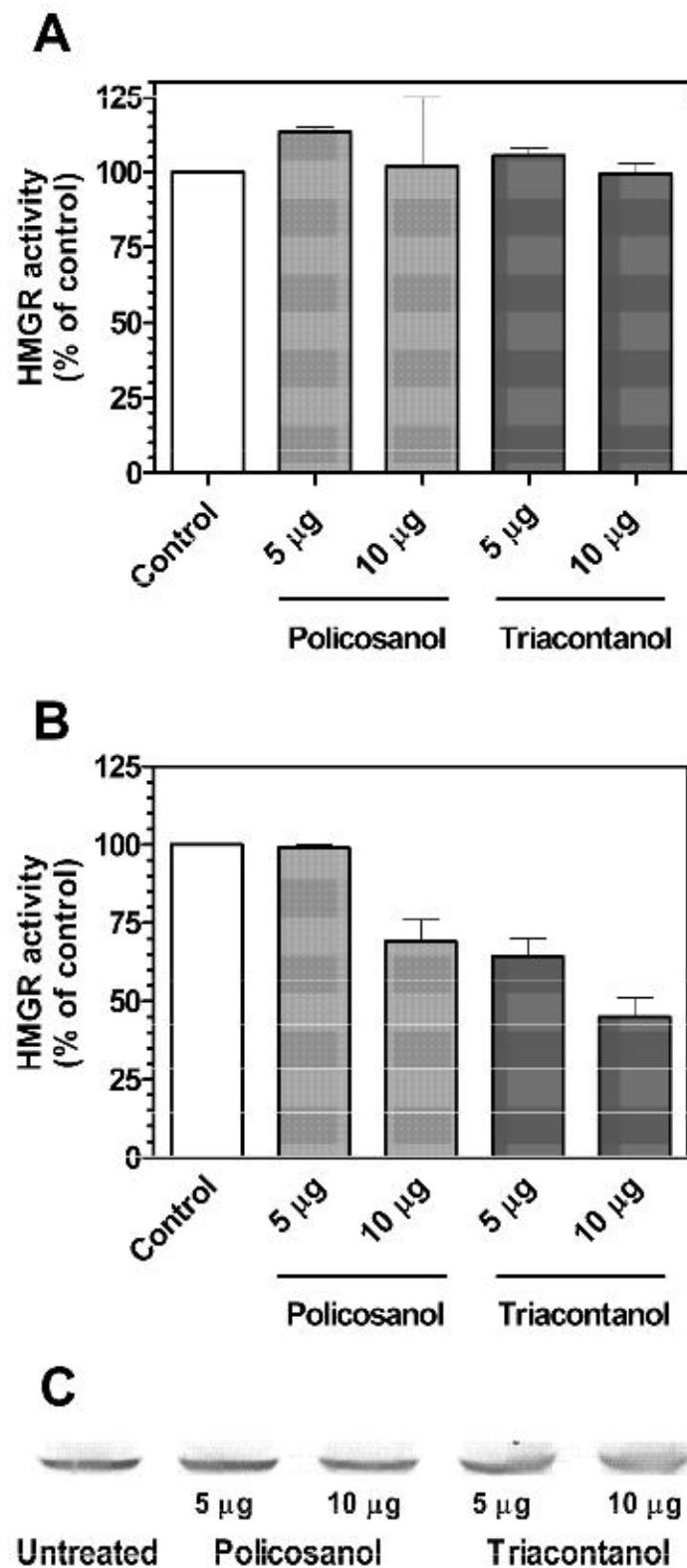


Fig. 6

