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**PRODUCTO NATURAL**

# *In vitro* effects of policosanol (*Saccharum officinarum* L wax alcohols) on the 5-lipooxygenase enzyme

## Efecto *in vitro* del policosanol (alcoholes de la cera de *Saccharum officinarum L*) sobre la enzima 5-lipooxigenasa

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**ABSTRACT**

**Introduction:** policosanol, a mixture of high molecular weight aliphatic alcohols purified from sugarcane with octacosanol as the main component, shows cholesterol-lowering and antiplatelet effects in addition to an inhibitory effect on type I cicloxygenase.
**Objective:** to determine whether policosanol may inhibit 5-LOX enzyme activity *in vitro*.
**Methods**: effects on 5-LOX enzyme activities were assessed in rat blood polymorphonuclear leukocytes. Vehicle or Policosanol suspensions (0.6 to 6 000 µg/mL) were added to tubes containing the reaction mix and then absorbance changes at 234 nm were measured.
**Results:** added Policosanol inhibited *in vitro* 5-LOX activity by 30 %, which was not a significant figure but depended on the concentration ($r = 0.992$; $p < 0.05$); it was 1 250 µg/mL.
**Conclusions:** policosanol did not significantly inhibit 5-LOX enzyme activity in rat PMNL preparations, so that it does not seem to be a dual inhibitor of COX and-LOX enzymes. This result differs from that found for beeswax alcohols and underlines the different effects of the mixtures of long-chain fatty alcohols purified from the sugarcane and the beeswax.

**Key words:** policosanol, sugarcane wax alcohols, lipoxygenases, 5-LOX, *Saccharum officinarum L.*

**RESUMEN**

**Introducción:** el policosanol es una mezcla de alcoholes alifáticos aislados y purificados de la caña de azúcar cuyo componente mayoritario es el octacosanol, con efecto sobre la reducción de colesterol y antiagregante plaquetario, además inhibe la ciclooxigenasa (COX) tipo 1.
**Objetivo:** determinar el poder de inhibición del policosanol en la actividad de la enzima 5-LOX *in vitro*.
**Métodos:** el efecto sobre la actividad de la enzima 5-LOX se determinó en leucocitos polimorfonucleares obtenidos de sangre de ratas. Se añadieron vehículo o suspensiones de policosanol (0,6 a 6 000 µg/mL) a tubos que contenían la mezcla de reacción y se medió el cambio de absorbancia a 234 nm.
**Resultados:** la adición de policosanol inhibió *in vitro* la actividad de la 5-LOX en un 30 % que no fue significativo pero sí dependiente de la concentración ($r = 0,992$; $p < 0,05$), inhibición esta que alcanzó 1 250 µg/mL.
**Conclusión:** el policosanol no inhibió significativamente la actividad de la enzima 5-LOX en preparación de polimorfonucleares de ratas, por lo que no es un inhibidor dual de las enzimas. Este resultado difiere del encontrado para los alcoholes de la cera de abeja y subraya la diferencia de los efectos hallados entre las mezclas de alcoholes alifáticos de cadenas largas purificados de la caña de azúcar y la cera de abeja.

**Palabras clave:** policosanol, alcoholes de la cera de caña de azúcar, lipooxygenasas, 5-LOX, *Saccharum officinarum L.*

## INTRODUCTION

Policosanol is a mixture of eight high molecular weight aliphatic alcohols purified from sugar cane (*Saccharum officinarum*, *L*) wax, wherein octacosanol ($C_{28}$) (60-70 %) is the main component, and $C_{24}$, $C_{26}$, $C_{27}$, $C_{29}$, $C_{30}$ , $C_{32}$ and $C_{34}$ alcohols are at lower concentrations.[1] In turn, D-002 is a mixture of six high molecular weight aliphatic alcohols ($C_{24}$, $C_{26}$, $C_{29}$, $C_{30}$, $C_{32}$, $C_{34}$) purified from beeswax that contains triacontanol ($C_{30}$) as the most abundant component.[2]

Policosanol has been reported to inhibit cholesterol synthesis through a regulation of HMG-CoA reductase[3] that involves the phosphorylation of AMP-kinase and HMG-CoA reductase, a mechanism demonstrated in hepatoma cells and in mouse liver after intragastric administration.[4,5] Also, it significantly raises LDL receptor-dependent processing, increasing LDL catabolic rate.[6] Recent data support that policosanol inhibits cyclooxygenase-1 (COX-1) activity *in vitro*,[7] which could explain, at least partly, its antiplatelet effects.[8,9] This inhibition of 5-LOX by D-002 should also explain, at least partly, its antioxidant effects on lipid peroxidation, but such inhibition has not been demonstrated for policosanol.

In light of these issues, this study was undertaken to investigate whether policosanol may inhibit 5-LOX activity *in vitro*.

## METHODS

### ANIMALS

Male Wistar rats (180-200 g) purchased from the Centre for Laboratory Animals Production (CENPALAB, Habana, Cuba) were adapted for 7 days to laboratory conditions: controlled temperature 25 ± 2 ˚C, relative humidity 60 ± 5 % and 12 h light/dark cycles. Food (rodent pellets from CENPALAB) and water were provided *at libitum*.

After a 12 h fast rats were anaesthetized in ether atmosphere, sacrificed by exsanguinations. We studied the effect on 5-LOX enzyme activity by using the cytosolic fraction of rat polymorphonuclear leukocytes (PMNL) prepared from total blood samples.

The study was conducted in accordance with the Cuban Guidelines for the laboratory animals care and Good Laboratory Practices. An independent ethic board for animal use approved the protocol of this study.

### MATERIALS

All chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO), except 2,2 azo-bis-2-amidinopropane hydrochloride (ABAP), obtained from Polyscience (Warington, PA). Ultracentrifuge was from Beckman (Beckman Instruments, Inc. Palo Alto, CA) and Utrospec-Plus spectrophotometer from LKB (Pharmacia LKB Biotechnology, Uppsala, Sweden).

### ADMINISTRATION AND DOSAGE

Policosanol was obtained from the Plant of Natural Products (National Centre for Scientific Research, Havana City, Cuba), after corroborate that they met the quality criteria for batch releases. The composition of the batch, assessed with a validated gas chromatographic method,[10] was as follows: tetracosanol 0.07 %, hexacosanol 4.9 %, heptacosanol 0.8 %, octacosanol 63.8 %, nonacosanol 0.5 %, triacontanol 12.8 %, dotriacontanol 6.8 %, tetratriacontanol 2.4 %. Policosanol concentrations were prepared as suspensions in Tween 20/water (2 %) vehicle. Nordihydroguaiaretic acid (NDGA) (Merck, Germany), the 5-LOX reference inhibitor, was dissolved in carboxymethyl-cellulose (2 %).

### PREPARATION OF THE POLYMORPHONUCLEAR LEUKOCYTES (PMNL) CYTOSOLIC FRACTION

The effects on 5-LOX activity were assessed by using enzyme preparations from the cytosolic fraction of rat blood polymorphonuclear leukocytes (PMNL) freshly isolated.[11] In brief, venous blood samples were collected in tubes containing EDTA (10 %) and diluted to 10 mL with the same volume of 0.9 % NaCl (saline) solution.

Then 6 ml of diluted blood were gently layered over 3 ml of 14.1 % Nycodenz (density 1.077 g/ml, 20 ºC) prepared in 0.44 % NaCl and 5 mmol/L Tris HCl buffer (pH 7.2), and centrifuged at 800 × g for 30 min. at 20 °C. After centrifugation, the mononuclear cells formed as band at the Nycodenz-plasma interface were removed with a Pasteur pipette, washed with 50 mmol/L phosphate buffer/1 mmol/L EDTA (pH 7.4), and centrifuged at 400 × g for 10 min. The pellet was washed again with the same buffer, re-suspended in the same buffer and then used as the crude enzyme preparation.

PMNL were then sonicated (3 cycles of 30 s, sub-maximal potency), centrifuged at 2000 × g for 10 min at 0 ˚C, and the supernatant centrifuged at 100 000 × g for 1 h at 4 ˚C. The cytosolic fraction was then frozen at -20 °C to use.

### EFFECTS ON 5-LOX ENZYME ACTIVITY

Lipoxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1-4 diene structures. The conversion of linoleic acid to 13-hydroperoxy linoleic acid was followed spectrophotometrically by the appearance of a conjugate diene at 234 nm on a UV/visible spectrophotometer.[11] In brief, the enzyme preparation (1 ml, final volume) that contained the cytosolic fraction (50 µg of protein) dissolved in 50 mmol/L phosphate buffer/1mmol/L EDTA (pH 7) was pre-incubated for 5 min prior to add the substrate (linoleic acid 250 µmol/L in ethanol).

Parallel tubes containing the vehicle (2 % Tween-20/$H_2O$), policosanol (0.6, 4.8, 19.5, 78.1, 312.5, 1 250, 2 500, 5 000, 5 500, 6 000 µg/mL), or NDGA (50 µg/mL) were run. Once the substrate was added, the increase of absorbance at 234 nm was measured every min for 10 min in the spectrophotometer. The enzyme activity was expressed as mmol of conjugated dienes/min/mg protein.

### STATISTICAL ANALYSES

Data were expressed as the mean ± SD. Comparisons between treated and control groups were performed with the Mann-Whitney U tests. Statistical significance was chosen for $\alpha = 0.05$. Dose-effect relationship was assessed by using a linear regression and correlation test. Regression analysis was used to calculate $IC_{50}$, defined as the concentration of inhibitor necessary for 50 % inhibition of the enzyme reaction. Data were processed with the Statistics Software for Windows (Release 4.2 Stat Soft Inc, Tulsa OK, US).

## RESULTS

Table summarizes the effects on 5-LOX activity. The addition of the 5-LOX inhibitor (NDGA 50 mg/ml) inhibited significantly and markedly the enzyme activity by 88 %.

Policosanol addition dose-dependently ($r = 0.992$; $p < 0.05$), but not significantly, inhibited *in vitro* 5-LOX activity to approximately 30 %, achieving this mild ceiling effect with a concentration of 1 250 µg/mL.

**Table.** Effect of policosanol on 5-LOX enzyme activity on rat PMNL cytosolic fraction

| Concentrations (µg/mL) | Enzyme activity (µmol of conjugated dienes/min/mg protein) | Inhibition (%) |
|---|---|---|
| Control | 7.40 ± 0.005 | - |
| NDGA 50 | 0.89 ± 0.251*** | 88.0 |
| Policosanol | | |
| 0.6 | 7.10 ± 0.009 | 4.0 |
| 1.2 | 6.90 ± 0.003 | 6.7 |
| 4.8 | 6.60 ± 0.001 | 10.8 |
| 19.5 | 6.30 ± 0.001 | 14.8 |
| 78.1 | 6.00 ± 0.001 | 18.9 |
| 312.5 | 5.70 ± 0.005 | 22.9 |
| 1 250 | 5.30 ± 0.001 | 28.3 |
| 5 000 | 5.20 ± 0.002 | 29.7 |
| 5 500 | 5.10 ± 0.001 | 30.0 |
| 6 000 | 5.10 ± 0.001 | 30.0 |

(Mean ± SD) ***p< 0.001, Comparison with the control (Mann Whitney U test).
PMNL: polymorphonuclear leukocytes;
5-LOX: 5-lipooxigenasa; NDGA: Nordihydroguaiaretic acid.

## DISCUSSION

This study demonstrates that the addition of policosanol (0.6-6 000 µg/mL) did not inhibit significantly or meaningfully *in vitro* 5-LOX activity in rat PMNL.

As expected, the addition of NDGA 50 µg/mL, a 5-LOX inhibitor used as reference, inhibited significantly and markedly (88 %), a result that confers validity to our experimental conditions for the testing of 5-LOX enzyme activity and to the present results obtained in such conditions.

Although policosanol produced a concentration-dependent reduction of 5-LOX activity up to 30 %, which suggests some action on this target, it failed to inhibit it significantly. This result diverges from that found for D-002, a mixture of beeswax alcohols that contains octacosanol, which inhibited both 5-LOX and COX enzyme activities, with a greater affinity for 5-LOX.[12] In fact, since recent studies have demonstrated that octacosanol, the main component of policosanol,[13] produces antinflammatory effects *in vivo*,[14,15] and policosanol inhibits COX-1 activity,[8] but it does not produce gastrotoxicity,[1,14-16] we expected that policosanol should inhibit 5-LOX enzyme, so that this effect could minimize the gastrotoxicity induced by COX inhibition.[16]

In fact, inflammation involves high levels of arachidonic acid (AA) from damaged cell membrane phospholipids generated through the activity of phospholipase A2 enzyme, which is then metabolized through the COX and LOX pathways to produce mediators like prostaglandins, thromboxanes, prostacyclins, and highly inflammatory leukotrienes (LT). COX inhibitors curtail prostaglandins formation, which turn the AA metabolism towards the LOX pathway, increasing the production

of LT and enhancing the toxicity due to the prostaglandins deficit, as demonstrated the high concentrations of $LTB_4$ seen in gastric ulcers induced by COX inhibitors, which attract leukocytes to the stomach and contributes to produce the ulceration.[17,18]

Nevertheless, this study was conducted *in vitro*, so that we cannot exclude that policosanol may inhibit 5-LOX *in vivo*. In such regard, we should remember that policosanol and octacosanol administered orally,[19,20] but not added *in vitro*,[21] exhibits antioxidant effects. Then, may be such effect requires, as the activation of AMP kinase and subsequent inhibition of HMGCoA reductase,[3,4] the metabolism of fatty alcohols to acids. Further studies, therefore, should explore the possibility that policosnol can inhibit 5-LOX *in vivo*.

## CONCLUSIONS

*In vitro* addition of Policosanol (0.6 to 6 000 µg/mL) did not inhibit significantly 5-LOX enzyme activity in rat PMNL preparations, so that it does seem to be a dual inhibitor of COX and-LOX enzymes. This result diverges of that found for beeswax alcohols and underlines the different effects of the mixtures of long-chain fatty alcohols purified from sugarcane and bees waxes.

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