Research Article

Effects of Policosanol (Sugar Cane Wax Alcohols) and D-003 (Sugarcane Wax Acids) on Cyclooxygenase (Cox) Enzyme Activity *In Vitro*

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ABSTRACT

Both policosanol and D-003 are mixtures of higher aliphatic alcohols and acids, respectively, purified from sugarcane wax, whose main components are octacosanol and octacosanoic acid (an active metabolite of octacosanol), respectively. Both substances share some, not all, pharmacological effects, like cholesterol-lowering, antiplatelet and antioxidant effects. The mechanisms whereby they exhibit cholesterol-lowering have been investigated, but those supporting their antiplatelet effects remain unknown. We hypothesized that these substances could inhibit cyclooxygenase (COX) activity. The aim of this study was to investigate whether policosanol and D-003 may inhibit COX-1 and COX-2 enzyme activities, effects assessed in cytosolic microsomes from rat platelets and seminal vesicles, respectively. Vehicle, Policosanol or D-003 suspensions (0.6 to 6000 μg/mL) were added to tubes containing a mixture of reactions and absorbance changes at 480 nm were measured. Both policosanol and D-003 inhibited significantly (maximal inhibition ≅ 80%) and dose-dependently COX-1 enzyme activity (IC_{50} = 312.5 μg/ml and 14.84 μg/ml, respectively) by modifying both kinetic parameters, so that the inhibition was uncompetitive. D-003, not policosanol, also inhibited significantly and dose-dependently, but moderately (maximal inhibition ≅ 54%), COX-2 activity. Concluding, policosanol and D-003 produce marked and comparable *in vitro* inhibitions of COX-1 activity in rat platelet microsomes, D-003 being the most potent. D-003, not policosanol, also inhibits moderately COX-2 activity in rat seminal vesicles.

Keywords: Cicloxygenases, COX-1, COX-2, D-003, Policosanol, sugarcane wax acids.

INTRODUCTION

Policosanol is a mixture of high molecular weight aliphatic alcohols purified from sugar cane (*Saccharum officinarum*, *L*) wax that contains octacosanol (C_{28}) as the main component, while C_{24}, C_{26}, C_{27}, C_{29}, C_{30}, C_{32} and C_{34} alcohols are at lower concentrations,[1] while D-003 is a mixture of higher aliphatic acids purified from the same source that contains C_{24}, C_{25}, C_{26}, C_{27}, C_{28}, C_{29}, C_{30}, C_{31}, C_{32}, C_{33}, C_{34}, C_{35} and C_{36} acids, wherein octacosanoic (C_{28}) acid, an active metabolite of octacosanol,[2] is the most abundant compound. [3]

Cholesterol-lowering effects of policosanol and D-003 have been demonstrated in experimental and clinical studies.[1-14] Policosanol has been reported to inhibit cholesterol synthesis through a regulation of HMG-CoA reductase that involves the phosphorylation of AMP-kinase,[15] a mechanism demonstrated in hepatoma cells and in mouse liver after intragastric administration.[16-18] Nevertheless, the activation of AMP-kinase by policosanol requires peroxisomal metabolism of the long chain alcohols that compose policosanol to produce the corresponding fatty acids.[18] Also, policosanol significantly increases LDL receptor-dependent processing, increasing LDL catabolic rate.[4]

Although the mechanism of the cholesterol-lowering effect of D-003 has been less studied than that of policosanol, it is known that D-003 also inhibits cholesterol synthesis by regulating HMG-CoA reductase activity *in vitro*[19] and that it increases LDL catabolic rate *in vivo*.[20] Keeping in mind these facts and that D-003 contains octacosanoic acid, an active metabolite of octacosanol, it is plausible that its mechanism also involves the regulation of HMG-CoA reductase by activating AMP-kinase, but this matter has not been proven.

On the other hand, policosanol and D-003 have shown to inhibit platelet aggregation[21-27] and to decrease serum thromboxane B_2 (TxA_2) levels.[16,17] Nevertheless, the mechanism of the antiplatelet effects of these substances remains unknown. Considering the key role of cyclooxygenase-1 (COX) enzyme in platelet aggregation we hypothesized that policosanol and D-003 should inhibit COX-1 enzyme as a plausible mechanism of their antiplatelet effects. To explore the selectivity of such effect, if any, we investigated their effects on both COX-1 and COX-2 enzyme activities.

In light of these issues, this study investigated whether policosanol and D-003 may inhibit COX (COX-1 and COX-2) enzyme activities.

MATERIALS AND METHODS

Animals

Male Wistar rats (180 – 200g) 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 hours light/dark cycles. Food (rodent pellets from CENPALAB) and water were provided *at libitum*.

After a 12 hour fast rats were anaesthetized in ether atmosphere, sacrificed by exsanguinations. Effects on COX-1 and COX-2 activities were assessed in cytosolic microsomal preparations from rat platelets and rat seminal vesicles, respectively.

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, The Havana, Cuba), after corroborate that they met the quality criteria for batch releases. The composition of the policosanol batch, assessed with a validated gas chromatographic method [28] 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%. In turn, D-003 contained tetracosanoic 0.4%, pentacosanoic 0.3%, hexacosanoic 1.0%, heptacosanoic 3.1%, octacosanoic 57.0%, nonacosanoic 3.0%, triacontanoic 20.0%, hentriacontanoic 1.2%, dotriacontanoic 12.1%, tritriacontanoic 2.0%, tetratriacontanoic 12.0%, pentatriacontanoic 0.5%, hexatriacontanoic 6.1%. This composition was determined by using a specific gas chromatographic method for this substance. [29]

Policosanol and D-003 were given as suspensions prepared in Tween 20/water (2%) vehicle. Indomethacin (Cuban Pharmaceutical Industry –QUIMEFA-) was dissolved in 5% sodium bicarbonate.

Preparation of the rat platelets microsomal fraction

The effects on COX-1 activity were assessed by using enzyme microsomal preparations from rat platelets. In brief, venous blood samples were collected in tubes containing sodium citrate (38%) 9:1 (v/v). The tubes were centrifuged at 160 x g for 10 min at 10 ^{o} C and the supernatant was centrifuged again at 2100 x g for 10 min at 10 ^{o} C. The pellet was re-suspended in Tris-HCl EDTA (50 mol/L, pH 7.4, 1 mol/L EDTA) and ammonium oxalate (2%) 1/20 (v/v) and centrifuged at 2100 x g for 10 min at 4^{o}C. The pellet was re-suspended again the same Tris-HCl EDTA buffer, sonicated (3 cycles of 30 sec, sub-maximal potency) and centrifuged at 15000 x g for 20 min at 4 ^{o} C. Finally, the supernatant was centrifuged at 100 000 x g for 2 hours at 4 ^{o} C. The pellet (platelets microsomal fraction) was re-suspended in 0.05 mol/L Tris/HCl buffer (pH 8.4) containing 0.01% Triton X-100) 1:9 (p/v) and frozen at-20^{o}C until use. [30]

Preparation of the rat seminal vesicles microsomal fraction

The effects on COX-2 activity were assessed by using microsomal preparations from rat seminal vesicles. In brief, seminal vesicle slices were homogenized in 0.05 mol/L Tris/HCl buffer (pH 8.4) containing 0.01% Triton X-100) 1:9 (p/v) with a potter. The homogenates were centrifuged at 15000 x g for 15 min and the supernatant was centrifuged again at 100000 x g for 1 hour, all operations being carried out at 4^{o}C, the pellet (microsomal fraction) was frozen at-20^{o}C until use. [31]

Effects on COX enzyme activity

COX activity was measured in accordance to Abad et al., 1994.[31] The reaction mixture contained 2 mmol/L arachidonic acid (AA); microsomal fraction (1 mg/ml); 5.8 mmol/L L-epinephrine and 0.05 mol/L Tris HCl) buffer(pH 8.4). Tubes containing the vehicle, policosanol or D-003 (0.6, 4.8, 19.5, 78.1, 312.5, 1250, 2500, 5000, 5500, 6000 μg/ml), or indomethacin (0.4 μg/ml) (reference inhibitor) were run in parallel. Then, mixture reactions were pre-incubated with L-epinephrine for 4 min first and then AA was added at 37^{o}C. The changes of optical density (O.D.) at 480 nm were measured for 10 min in the spectrophotometer. The enzyme activity was expressed as the changes of O.D./mg of protein.

Each experiment was run in triplicate and the results averaged. The concentration producing a 50% inhibition (IC_{50}) was calculated from the outline of the inhibition percentages as a function of the concentrations of the intended inhibitors (policosanol or D-003). The effects on COX reaction rates were assessed in front of increasing concentrations of the substrate (AA 7.8, 31.2, 62.5, 125, and 250 mmol/L).

Once the substrate was added, we measured the increase of D.O. at 234 nm per min for 10 min in the spectrophotometer. The enzyme activity was expressed as μmol of conjugated dienes/min/mg protein. The initial reaction rate was determined from the slope of the straight line portion of the curve and the percentage inhibition of the enzyme activity was calculated by comparing with the control samples.

Statistical analyses

Data were expressed as the mean ±SD. Comparisons between treated and control groups were performed with the Kruskal-Wallis and the Mann-Whitney U tests. Statistical significance was chosen for α= 0.05. Dose-effect relationships were 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 1 summarizes the effects on COX-1 activity. The addition of policosanol (0.6 - 6000 µg/ml), inhibited COX-1 activity (r= 0.993; p< 0.05) (IC_{50} = 312.5 µg/ml) significantly and dose-dependently. A maximal inhibition of about 80% was achieved with 5500 µg/mL. In turn, D-003 addition (0.6 - 6000 µg/ml) also produced a significant and dose-dependent inhibition of COX-1 (r=0.971; p< 0.05) (IC_{50} = 14.84 µg/ml) (maximal inhibition, reached with 1250 µg/mL, was approximately 80%).

Table 1: Effects of policosanol and D-003 on COX-1 enzyme activity on rat platelets microsomal fraction

Concentrations (µg/ml)	Enzyme activity (ΔOD/min/mg protein)	Inhibition (%)
Control (0)	0.350 ± 0.001	-
Policosanol		
0.6	0.316 ± 0.003	10
1.2	0.275 ± 0.001	21
4.8	0.258 ± 0.001	26
19.5	0.212 ± 0.005*	39
78.1	0.188 ± 0.002*	46
312.5	0.147 ± 0.001**	58
1250	0.138 ± 0.005**	61
5000	0.089 ± 0.004**	75
5500	0.077 ± 0.001**	78
6000	0.077 ± 0.001**	78
D-003		
0.6	0.279 ± 0.006	20
1.2	0.240 ± 0.034*	32
4.8	0.192 ± 0.067*	45
19.5	0.169 ± 0.036*	52
78.1	0.137 ± 0.052*	61
312.5	0.086 ± 0.048*	75
1250	0.077 ± 0.002**	78
5000	0.076 ± 0.004**	78
5500	0.069 ± 0.001**	80
6000	0.071 ± 0.001**	80
Indomethacine	0.059 ± 0.003***	83

(Mean ± SD) O.D. Optical density; *p<0.05, **p<0.01, ***p<0.001, Comparison with the control (Mann Whitney U test)

The inhibitory effects of policosanol and D-003 involved the modification of both kinetic parameters (Vmax and Km) of COX-1 activity (Figures 1 and 2, Lineweaver-Burk plots), therefore, the inhibition was uncompetitive.

Table 2 summarizes the effects on COX-2 activity. Although policosanol produced a moderated and dose-dependently reduction of COX-2 activity (r= 0.972; p< 0.05), the comparisons with the control were not significant and the inhibition (39%) achieved with the highest dose (6000 µg/ml) was below the criterion to be considered as an effective enzyme inhibitor.

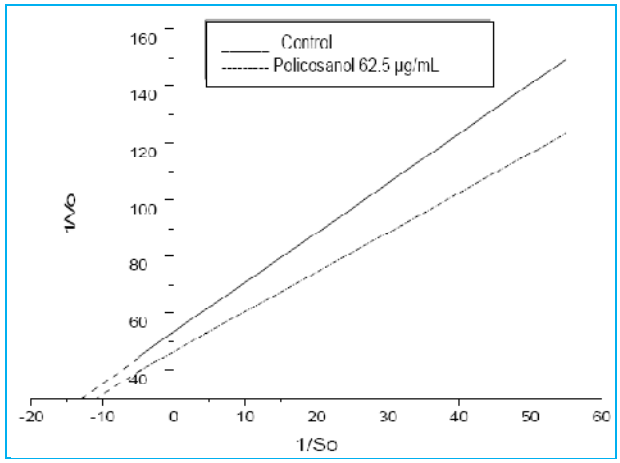

Figure 1: Lineweaver-Burk plot (1/v0 versus1/[S]0), of the effect of policosanol (62.5 µg/ml) on the initial rate of the enzyme reaction measured in front of increasing concentrations of the substrate (arachidonic acid 7.8, 31.2, 62.5, 125 and 250 mmol/L). Policosanol modified the values of both kinetic parameters Km (-1/Km, intercept with abscise axis) and Vmax (1/Vmax, intercept with the ordinate axis) of COX-1 enzyme activity.

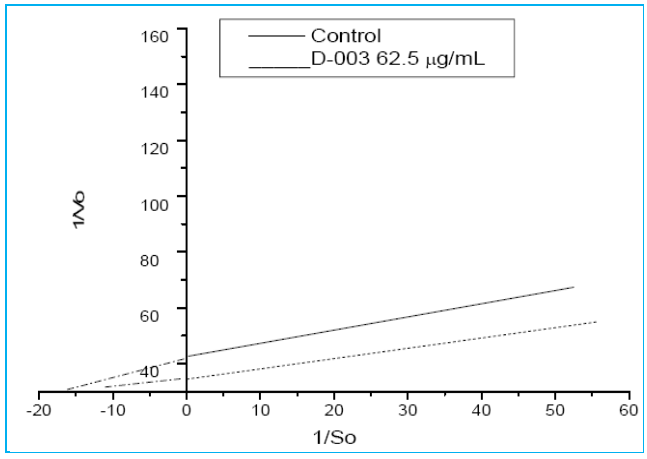

Figure 2: Lineweaver-Burk plot ($1/v_0$ versus$1/[S]_0$), of the effect of D-003 (62.5 µg/ml) on the initial rate of the enzyme reaction measured in front of increasing concentrations of the substrate (arachidonic acid 7.8, 31.2, 62.5, 125 and 250 mmol/L). D-003 modified the values of both kinetic parameters Km (-1/Km, intercept with abscise axis) and Vmax (1/Vmax, intercept with the ordinate axis) of COX-1 enzyme activity.

By contrast, D-003 inhibited significantly and dose-dependently (r=0.990; p< 0.05), although moderately (maximal inhibition ≅ 54%) COX-2 enzyme activity. This inhibition involved the modification of Vmax and Km (Lineweaver-Burk plot, data not shown for simplicity), so that it was uncompetitive. Indomethacin (0.4 µg/ml) inhibited COX-1 and Cox-2 inhibited enzyme activities by 83 and 89%, respectively.

Table 2: Effects of policosanol and D-003 on COX-2 enzyme activity on rat vesicle seminal microsomal fraction

Concentrations (µg/ml)	Enzyme activity (ΔOD/min/mg protein)	Inhibition (%)
Control (0)	0.350 ± 0.001	-
Policosanol		
0.6	0.341 ± 0.007	3
1.2	0.307 ± 0.003	12
4.8	0.288 ± 0.002	18
19.5	0.275 ± 0.005	21
78.1	0.249 ± 0.001	29
312.5	0.231 ± 0.001	34
1250	0.224 ± 0.001	36
5000	0.216 ± 0.001	38
5500	0.216 ± 0.001	38
6000	0.214 ± 0.001	39
D-003		
0.6	0.320 ± 0.015	9
1.2	0.290 ± 0.014	17
4.8	0.265 ± 0.039	24
19.5	0.248 ± 0.038	29
78.1	0.238 ± 0.005	32
312.5	0.225 ± 0.005	36
1250	0.193 ± 0.030	45
5000	0.163 ± 0.053*	53
5500	0.163 ± 0.055*	54
6000	0.161 ± 0.051*	54
Indomethacine	0.040 ± 0.001***	89

(Mean ± SD) O.D. Optical density; *p<0.05, **p<0.01, ***p<0.001, Comparison with the control (Mann Whitney U test)

DISCUSSION

This study demonstrates that the addition of policosanol and D-003 (0.6 - 6000 µg/ml) produce comparable *in vitro* inhibitions of COX-1 activity in rat platelet microsomes, D-003 being more potent than policosanol. D-003, not policosanol, also inhibits COX-2 activity in rat seminal vesicles, but moderately.

As expected, the addition of indomethacin 0.4 µmol/L, a non selective NSAID used as a reference inhibitor, inhibited both COX-1 and COX-2 by 83 and 89%, respectively. This result confers validity to our experimental conditions to test COX-1 and COX-2 enzyme activities and to the present results.

The inhibitions of COX-1 enzyme activity with policosanol and D-003 were marked, so that maximal inhibitions of about 80% were obtained with the two substances. Despite the fact that the inhibitory effects of both treatments were similar, D-003 was more potent than policosanol, as reflects their IC_{50} values (14.84 µg/ml and 312.5 µg/ml, respectively). This affirmation is also supported by the fact that the maximal inhibitory concentration of D-003 (1250 µg/ml) is about 4.4 times lower than that of policosanol (5500 µg/ml).

The inhibitory effects of policosanol and D-003 on COX-1 activity were uncompetitive since they modified the affinity for the substrate (Km) and the Vmax of the enzymatic reaction, which suggests that instead of interacting with the enzyme active site, they should interact with a site near to that in such a way that may curtail the enzyme reaction. The present study does not support more details of such interaction, which should be further investigated. Nevertheless, considering the long structures of high molecular weight alcohols and acids, their direct interaction with the enzyme active site should not be probable.

The remarkable effects of policosanol and D-003 on COX-1 as compared to COX-2 are consistent with their antiplatelet effects, as COX-1 enzyme isoform is the key enzyme for the formation of eicosanoids in platelets, where this enzyme is expressed.[32] In line with these results, significant reductions of serum TxB_2 (an inactive metabolite of thromboxane A_2) levels have been observed with policosanol and D-003. [24-26]

The effect of D-003 on COX-1 activity, however, was not specific because it also produced a significant and dose-dependent inhibition of COX-2 activity, although modest in magnitude (54% inhibition). This finding suggests that D-003 should act as a non selective NSAID that share antiplatelet and antiinflammatory effects, as other drugs of this class, but such hypothesis deserves the *in vivo* demonstration of the antiinflammatory action of D-003, a matter not explored yet.

On its side, policosanol produced a dose-dependent reduction of COX-2 activity up to 39% that suggests some inhibitory action on this target, but it failed to inhibit this enzyme activity significantly, so that we do not expect a clinically relevant antiinflammatory effect derived from its effect on COX-2.

The recent demonstration of the *in vivo* antiinflammatory effects of octacosanol, the main component of policosanol,[33] and of other mixtures of long chain alcohols, [34,35] suggests that policosanol should exhibit this effect. In such case, not demonstrated yet, such antiinflammatory effect, if any, should be ascribed mainly to the inhibition of COX-1 rather than to its mild and not significant effect on COX-2. Nevertheless, since this study investigated the effects on COX activities *in vitro*, not *in vivo*, we cannot exclude that policosanol may produce a meaningful inhibition of COX-2 *in vivo* as result from the metabolic transformation of the long chain fatty alcohols into fatty acids, as occurs in the case of cholesterol synthesis.[18] The present results merit, therefore, to explore the antiinflammatory effect of policosanol *in vivo*, and if any, its probable mechanism beyond COX-1 inhibition.

CONCLUSION

Policosanol and D-003 produce marked and comparable *in vitro* inhibitions of COX-1 activity in rat platelet microsomes, D-003 being more potent than policosanol. D-003, not policosanol, also inhibits COX-2 activity in rat seminal vesicles, but moderately. This study gives, for the first time, some clues of the antiplatelet mechanism of policosanol and D-003.

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