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Synergistic effect of D-003 and aspirin on experimental thrombosis models

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Abstract

D-003 is a mixture of higher primary aliphatic saturated acids purified from sugarcane wax, with antiplatelet and antithrombotic effects experimentally demonstrated. Octacosanoic acid is the main component of D-003, followed by triacontanoic, dotriacontanoic, and tetracontanoic acids, while other acids are minor components. This work investigates the effects of combination therapy D-003 + aspirin (ASA) on arachidonic acid (AA)-induced sudden death in mice and bleeding time in rats. In addition, the effects of D-003 on serum levels of two metabolites of AA: thromboxane A_2 and prostacyclin, assessed through the measurement of their stable metabolites: thromboxane B_2 (TxB_2) and 6 keto PgF1α by radioimmunoassay kits, were also investigated. Combination therapy of D-003 (50 mg/kg) and ASA (3 mg/kg) significantly increased bleeding time in rats in a synergistic manner compared with D-003 or ASA alone. Moreover, the combined treatment of D-003 (200 mg/kg) and ASA (5 mg/kg) in mice protected against AA-induced sudden death (83% survivors) in a synergistic manner which was compared with each treatment alone (33% survivors). These results indicate that antiplatelet effects of D-003 are not mediated by a cyclooxygenase inhibition. D-003 and ASA monotherapies reduced serum TxB_2 levels, whereas D-003, but not ASA, significantly increased 6 keto PgF1α levels.

1. Introduction

The platelets play a central role in the pathogenesis of atherosclerosis and subsequent ischemic events [1]. Indeed, antiplatelet therapy is effective in the secondary prevention of cardiovascular events [2].

ASA is the antiplatelet drug most widely studied and considered as the standard of antiplatelet therapy. However, the discovery that ASA inhibited the synthesis of the prostacyclin (PgI_2) by vessel wall [3] raised the question of whether ASA's beneficial inhibition of platelet aggregation might in part be negated by the effect on PgI_2 synthesis. A further complicating factor has been introduced by the observation that salicylate, a product derived from the hydrolysis and enzymatic degradation of ASA, appears to compete with the active parent substance on the cyclooxygenase (COX) enzyme and antagonizes ASA both in the platelet and the vessel wall [4,5]. Therefore, newer agents acting at different stages of the platelet activation pathway have been developed, and they are therapeutically used in dependence of the characteristics of a given patient. However, the possibility that the antithrombotic effect of ASA could be improved by concomitant therapy with other drugs cannot be discarded.

D-003 is a mixture of high molecular weight primary aliphatic acids isolated and purified from sugarcane (*Saccharum officinarum* L.) wax, whose main component is 1-octacosanoic acid ($C_{28}H_{56}O_2$; 45.0–60.0%), followed by 1-triacontanoic ($C_{30}H_{60}O_2$, 15.0–30.0%), 1-dotriacontanoic ($C_{32}H_{64}O_2$; 10.0–25.0%) and 1-tetratriacontanoic acids ($C_{34}H_{68}O_2$; 10.0–25.0%). Also, 1-hexatriacontanoic ($C_{36}H_{72}O_2$), 1-hexacosanoic ($C_{26}H_{52}O_2$), 1-nonacosanoic ($C_{29}H_{58}O_2$), 1-tritriacontanoic ($C_{33}H_{66}O_2$), 1-hentriacontanoic ($C_{31}H_{62}O_2$), and 1-pentatriacontanoic ($C_{35}H_{70}O_2$; 0.5–1.5%) acids are present as minor ($\leqslant 5\%$) components [6]. D-003 has shown antiplatelet, antithrombotic [8] and cholesterol lowering [7] effects in experimental models.

The pharmacological profile of D-003 resembles that of policosanol, a mixture of higher primary aliphatic alcohols purified from the same source, but with a composition closely related, but different from that of

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D-003. Thus, 1-octacosanol is the major component of policosanol, followed by 1-triacontanol, 1-hexacosanol, 1-tetratriacontanol, 1-dotriacontanol, 1-heptacosanol y 1-nonacosanol and 1-tetracosanol [9] Policosanol shows cholesterol lowering and concomitant antiplatelet effects demonstrated in experimental models, healthy volunteers and patients with hypercholesterolemia type II [10–17].

The relation of D-003 and policosanol is not only based on the fact that both substances are isolated and purified from sugarcane wax, but also from their close structural and metabolic relationships. Thus, very long fatty acids are not only structurally, but also metabolically related to their corresponding alcohols by their mutual conversion, as demonstrated in cultured tissues [18]. In addition, octacosanoic acid, the main component of D-003, is not only formed in the liver from octacosanol [19–21], the main component of policosanol, but also it is an active metabolite derived from policosanol.

A positive interaction between policosanol and ASA on ex vivo and in vivo platelet aggregation models has been found, suggesting a mechanism of action different from COX inhibition [11,17]. In such regard, the reduction of thromboxane A_2 (TxA_2) and increased PgI_2 levels induced by policosanol in rodents could be the basis of its antiplatelet action [11–16]. Hence, it is logical to expect that D-003 could act by a mechanism similar to policosanol.

In addition, the reactive oxygen species play an important role in the platelet aggregation [22,23] and policosanol [24] and D-003 [25] possesses antioxidant effects.

The similarity in the effects of both drugs is supported not only by their closely related structures, but by the fact that octacosanoic acid, the main acid component of D-003, is the active metabolite of policosanol [26]. Nevertheless, some differences in the effects of both drugs have been observed. D-003 acts faster than policosanol and D-003, but not policosanol, increases bleeding time in experimental animals [8].

The aim of this work was to investigate the possible interaction of D-003 with ASA and the possible effects of both substances on serum thromboxane B_2 (TxB_2) and 6 keto PgF1α levels.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (250-300 g) and OF1 mice (25–30 g) were supplied by the National Center for Laboratory Animals (CENPALAB, Havana, Cuba). The animals were adapted to laboratory conditions for 7 days with free access to food and water.

2.2. Administration and dosage

D-003 and policosanol were supplied by the Chemistry Department of the Center of Natural Products (National Center for Scientific Research, Havana City, Cuba), and both were suspended in Tween 20–water (2%) vehicle. ASA was dissolved in sodium bicarbonate solution (5%).

D-003, was administered orally by gastric gavage (1 ml/100 g body weight) while control animals received equivalent volumes of the Tween 20–water (2%) vehicle. ASA (0.5 ml/100 g body weight) was administered orally by gastric gavage to rats or i.p. to mice.

2.3. Interaction study of D-003 and ASA on bleeding time in rats

2.3.1. Dose–effect relationship of ASA on bleeding time in rats

This assay was conducted to find the appropriate dose of ASA to investigate its possible interaction with D-003.

The animals were randomly distributed in four experimental groups: (1) a control group only receiving equivalent volumes of bicarbonate (5%) and Tween-20 (2%); and three groups treated with ASA (groups 2–4) as single doses of 1, 3, and 10 mg/kg. The two highest doses have been reported as effective in thrombosis models, being the dose of 3 mg/kg the minimum effective dose in rats [27]. Phil RB and Paul ML, 1983 [27] reported an effective reduction of platelet aggregation in rats obtained when this dose of ASA was used, while PgI_2 activity remained unchanged.

2.3.2. Interaction study of D-003+ASA on bleeding time in rats

Previous works have demonstrated that D-003 orally administered from 50 to 500 mg/kg increases significantly bleeding time in rats from 1 to 3 h after dosing.

On the other hand, the salicylates are absorbed rapidly, and notable plasmatic concentrations are found at 30 min after administration, while the maximal value is achieved at 2 h [28]. Moreover, the platelet COX inhibition with salicylates is irreversible during platelet half-life (7–10 days). [29] In consequence, the bleeding time was assayed at 90 min after ASA administration, as well as D-003.

The dose–effect relationship of ASA on bleeding time was determined. Later on, the animals were randomly distributed in four experimental groups: (1) a control receiving equivalents volumes of bicarbonate (5%) and Tween 20 (2%), (2) ASA at 3 mg/kg, (3) D-003 at 50 mg/kg and (4) ASA (3 mg/kg) + D-003 (50 mg/kg).

2.3.3. Bleeding time determination

Rats were anesthetized with sodium pentobarbital (40 mg/kg) i.p. The tail was immersed in an isotonic (0.9%) saline solution at 37°C for 5 min and then it was immediately cut off at 4 mm from the distal end. The bleeding time was recorded in seconds [30].

2.4. Interaction study of D-003+ASA on arachidonic acid (AA)-induced sudden death in mice

2.4.1. Dose–effect relationship of ASA on AA-induced death in mice

This assay was conducted to find the appropriate dose of ASA to be used in the interaction study with D-003.

The animals were randomly distributed in four experimental groups: (1) A control group only receiving equivalent volumes of bicarbonate (5%) and Tween-20 (2%); and three treated groups treated with single doses of ASA at 5, 15, and 50 mg/kg, since the highest doses have been reported as effective in thrombosis models in mice [31].

Previous works have reported that a single dose of D-003 (400 mg/kg but not 200 mg/kg) significantly protected from death induced by collagen plus epinephrine [32]. Therefore, 200 mg/kg of D-003 was chosen to use in combined therapy with ASA on AA-induced death in mice.

The dose–effect relationship of ASA on platelet aggregation was firstly determined. Later on, the animals were randomly distributed in four experimental groups: (1) a control, group receiving equivalent volumes of bicarbonate (5%) and Tween 20 (2%), (2) ASA at 5 mg/kg, (3) D-003 at 200 mg/kg and (4) ASA (5 mg/kg) + D-003 (200 mg/kg).

2.4.2. AA-induced sudden death in mice

One hour after treatment, mice were anesthetized with sodium pentobarbital (75 mg/kg) and AA sodium salt (Sigma) dissolved in saline solution (0.9%) at a concentration of 25 mg/ml by injecting into the dorsal penis vein (80 mg/kg). Animals living 60 min after the AA challenge were considered as survivors [33].

2.5. Effect of D-003 on serum TxB_2 and 6 keto PgF1α levels generated in the coagulation process in rats

The animals were randomly distributed in nine experimental groups: five groups for studying the effect of single doses and four for investigating the effect of repeated doses. At each experimental series, group 1 was constituted by control animals that received equivalent volumes of vehicle and D-003 treated groups (2, 3 and 4) (5, 25 and 200 mg/kg). In the single-dose approach, a group treated with ASA (10 mg/kg) was included.

D-003 and ASA were administered 90 min before the assay in the single-dose scheme, meanwhile in the repeated dose experience D-003 was administered during 10 days. The latest administration was performed 18 h before blood extraction to animals. In order to rule out any interference with drug absorption in this experiment, ASA was administered i.p. and D-003 by oral route.

2.5.1. Determination of serum of TxB_2 and 6 keto PgF1α levels generated in the coagulation process

The rats were anesthetized, their abdomen cavity opened and cava vein isolated for blood sampling. The blood samples (4 ml) were extracted by puncture and placed in glass tubes immediately introduced in a bath at 37°C during 1 h. Later on, the tubes were centrifuged at 2500 rpm for 10 min. The samples of serum supernatant were stored in Eppendorf tubes at 70°C.

Subsequently, serum TxA_2 and PgI_2 levels were determined using TxB_2 and 6 keto PgF1α radioimmunoassay kits (Amersham).

3. Results

Oral treatment with single doses of ASA (3–10 mg/kg) increased significantly the bleeding time in rats in dose-dependent manner. The dose of 1 mg/kg, however, was ineffective (Table 1).

Single oral doses of D-003 (50 mg/kg) and ASA 3 mg/kg similarly increased the bleeding time in rats. However, when both drugs were administered simultaneously, the bleeding time was significantly longer than that induced by D-003 or ASA alone. As can be observed, this combination therapy resulted more effective than ASA 10 mg/kg, a dose reported as the maximal in experimental thrombosis models in rats (Table 2).

Injection of AA into anesthetized mice induced death after 1 or 2 min in all control animals. Sudden death in mice was accompanied of respiratory arrest, convulsions and cardiac arrest. ASA pretreatment at 5, 15 and 50 mg/kg, i.p. protected animals significantly and dose dependently against AA-induced death (33.3%, 70% and 80% of the animals survived, respectively) (Table 3).

Table 1
Effect of ASA on bleeding time in rats

Treatment	Doses (mg/kg)	*N*	Bleeding time (s)	Increase (%)
Control		10	348.7±18.3	—
ASA	1	9	350.1±33.9	0.4
	3	9	480.0±54.9*	37.6
	10	9	552.3±34.7***	58.4

*$P<0.05$; ***$P<0.0001$. Comparison with the control group (Mann–Whitney *U* test).

Table 2
Effect of D-003, ASA and combined therapy ASA + D-003 on bleeding time in rats

Treatment	Doses (mg/kg)	Bleeding time (s)	Increase (%)
Control		325.1 ± 26.8	—
ASA	3	449.8 ± 37.5*	38.3
D-003	50	447.1 ± 40.2*	37.5
D-003 + ASA	50 + 3	606.8 ± 46.9***,†,‡	86.6

*$P<0.05$; ***$P<0.0001$. Comparison vs. control (Mann–Whitney U test).
†$P<0.05$. Comparison vs. ASA 3 mg/kg (Mann–Whitney U test).
‡$P<0.01$. Comparison vs. D-003 50 mg/kg (Mann–Whitney U test).

Table 3
Effect of ASA on AA-induced sudden death in mice

Treatment	Survivors/total	Survival (%)
Control (vehicle)	0/18	0
ASA (5 mg/kg)	6/18**	33
ASA (15 mg/kg)	7/10***	70
ASA (50 mg/kg)	8/10****	80

**$P<0.01$; ***$P<0.001$; ****$P<0.0001$. Comparisons vs. control group (Fisher's exact probability test).

Table 4
Effect of D-003, ASA and combination therapy ASA + D-003 on AA-induced sudden death in mice

Treatment	Survivors/total	Survival (%)
Control (vehicle)	0/18	0
ASA (5 mg/kg)	6/18**,†	33
D-003 (200 mg/kg)	4/12*,†	33
D-003 + ASA	10/12***	83.4

*$P<0.01$; **$P<0.001$; ***$P<0.0000$. Comparison vs. control. Fisher's exact test.
†$P<0.01$. Comparison vs. combined treatment. Fisher's exact test.

D-003 protected significantly against AA-induced sudden death also. At 200 mg/kg, 33.3% of the mice survived. Simultaneous pretreatment of mice with both drugs (ASA, 5 mg/kg, i.p. and D-003, 200 mg/kg, p.o.) resulted in a significant protection and reaching a survival rate of 83% that was significantly different from mice treated with ASA or D-003 alone (Table 4).

In this model however, combination therapy was not better than ASA administered as monotherapy at 50 mg/kg, which increase survival rate up to 80%.

Table 5 shows the effects of D-003 and ASA on levels of TxB_2 and 6 keto PgF1α. D-003 administered at 25 and 200 mg/kg as single oral dose reduced significantly serum TxB_2 levels, while only the highest dose significantly increased 6 keto PgF1α compared with control group. The dose of 5 mg/kg of D-003 resulted ineffective at all. Single dose of ASA at 10 mg/kg inhibited remarkably TxB_2 generation and reduced moderately 6 keto PgF1α (at the borderline of statistical significance ($P = 0.054$).

Table 5
Effect of D-003 on serum TxB_2 and 6 keto PgF1α levels generated in the coagulation process in rats

Treatment	6 keto PgF1α (ng/ml)	TxB_2 (ng/ml)	TxB_2/6 keto PgF1α
Single dose			
Control	4.8 ± 1.02	118.5 ± 15.4	43.8 ± 23.6
D-003 (5 mg/kg)	5.4 ± 0.8	87.5 ± 4.7	15.5 ± 1.9
D-003 (25 mg/kg)	6.7 ± 1.9	83.9 ± 5.1†	16.2 ± 2.08
D-003 (200 mg/kg)	9.2 ± 2.01†	82.8 ± 5.1†	9.9 ± 1.7†
ASA (10 mg/kg)	2.7 ± 0.4t	7.7 ± 3.4‡	4.08 ± 1.9†
Repeated doses			
Control	4.87 ± 1.13	81.5 ± 2.3	42.5 ± 27.1
D-003 (5 mg/kg)	6.10 ± 2.01	69.2 ± 8.9	15.7 ± 3.1
D-003 (25 mg/kg)	11.4 ± 2.5†	60.5 ± 6.3‡	7.3 ± 1.5‡
D-003 (200 mg/kg)	11.3 ± 2.5†	62.7 ± 6.2†	7.9 ± 1.8†

†$P<0.05$; ‡$P<0.0$; t = $P = 0.054$. Comparison with control.

D-003 at 25 and 200 mg/kg administered for 10 days significantly decreased TxB_2 and increased 6 keto PgF1α levels compared with control group, while at 5 mg/kg was ineffective.

Finally, the TxB_2/6 keto PgF1α ratio was significantly reduced after single dose of ASA at 10 mg/kg, D-003 at 200 mg/kg and repeated dose of D-003 at 25 and 200 mg/kg.

4. Discussion

A positive synergism between D-003 and ASA was evidenced in the two in vivo models used in the present study.

The combination therapy with D-003 (50 mg/kg) and ASA (3 mg/kg) prolonged significantly the bleeding time in rats, not only in relation to control group but also with respect to both drugs administered separately.

Furthermore, a simultaneous pretreatment of mice with ASA (5 mg/kg) and D-003 (200 mg/kg) resulted in a significant protection against AA-induced sudden death (83% survival). This protection was significantly higher than reached with respective monotherapies with ASA at 5 mg/kg or D-003 at 200 mg/kg which induced a survival rate of 33%, respectively.

In addition, this work has also demonstrated that single or repeated oral doses of D-003 reduced significantly TxB_2/6 keto PgF1α ratio by increasing serum 6 keto PgF1α (stable metabolite of PgI_2) levels and reducing serum TxB_2 (stable metabolite of TxA_2) levels, both quantified in serum after coagulation process. The effect induced by repeated doses was greater than that induced by single doses of D-003.

The effects of D-003 on AA metabolites and the observed synergism with ASA suggests that the

mechanism of the antiplatelet effect of D-003 is not mediated through COX inhibition.

Eicosanoids are AA derivatives formed in platelets and various parts of the vascular wall in a reaction catalyzed by COX. PgI_2 is mainly synthetized in endothelial cells by the additional action of PgI_2-synthase and exerts antiaggregant and vasodilatory effects. TxA_2 is mainly synthetized in platelets and only to a little extent in the vasculature. TxA_2 promotes platelet aggregation and vasoconstriction as well as smooth muscle cell proliferation. COX is the key enzyme for the formation of eicosanoids and can be blocked by substances such as indomethacin, ASA, ibuprofen or diclofenac [34].

Platelets, once formed and released from megakaryocytes, have lost their ability to synthetize proteins. When the COX enzyme is blocked, TxA_2-synthesis is subsequently discontinued for their life span. However, in the vascular wall, the synthesis of new enzyme proteins renders the inhibition of COX reversible. Thus, the effective balance between the counteracting effects of TxA_2 and PgI_2 is shifted towards the remaining actions of PgI_2 [35].

It has been demonstrated that the bleeding produced by cut of the last portion of tail of rat constitute an adequate experimental model wherein the hemorrhage and homeostasis are induced by mechanical damage [30].

It is well known that ASA induces a prolongation in the bleeding time by inhibiting the platelet function [36]. This action results from the irreversible inhibition of COX enzyme with the consequent inhibition of synthesis of TxA_2 in platelets and PgI_2 in vascular wall [37–39]. However, low doses of ASA are able to inhibit specifically the TxA_2 synthesis without affecting PgI_2 vessel wall production [26,36].

AA-induced sudden death in mice is a valid and useful in vivo model for the evaluation of antithrombotic drugs [33]. Platelet thrombi, trapped in the pulmonary microcirculation, are a key aspect of the sudden death [31,40]. TxA_2 is quantitatively a very prominent platelet eicosanoid product formed [41] and acts as a vasoconstrictor and potent proaggregatory agent. It was shown to be the major mediator of AA-induced sudden death in rabbits [42] and mice [43]. This model has been used to study the in vivo antithrombotic effects of COX inhibitors [44] among them ASA, which can practically protect all animals from death. This protective effect of ASA is related with its ability to abolish platelet aggregation induced by AA [45]. However, other drugs such as TxA_2 synthase inhibitors are effective in this model [46], drugs that not only inhibit TxA_2 generation, but also increase the production or release of PgI_2, thus counteracting the vasoconstriction and platelet aggregation induced by TxA_2.

The fact that D-003, in a similar manner as thromboxane synthase inhibitors, reduced significantly TxB_2/6 keto PgF1α ratio by increasing serum levels of 6 keto PgF1α and reducing serum levels of TxB_2 could explain the synergism with low dose of ASA observed in this study. This result is in concordance with other reports of synergism between ASA and antiplatelet drugs that act by mechanisms different from COX inhibition [47–49].

In conclusion, PgI_2 generated by the vessels wall could play an important role in the thrombosis protection on in vivo thrombosis models. The remarkable increasing of PgI_2 induced by D-003 could explain the synergistic effect between ASA and D-003.

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