Design, Synthesis and Antiinflammatory Activity of Novel γ-Tocopherol Acetylsalicylic acid Ester Codrug

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Abstract

Acetylsalicylic acid (ASA) is the most widely used drug for the prevention of thrombotic risk in the heart, brain and peripheral circulation. Different reports suggest that vitamin E decreases neuronal cell death caused by ischemia and reperfusion in rat models, and influences in vitro antiplatelet effect of aspirin. Unfortunately, ASA causes gastrointestinal side effects, due to its irreversible inhibition of the COX-1 in stomach. Moreover, the gastric side effect seems to involve an oxidative stress. For this reasons, the co-administration of vitamin E and ASA has been proposed to reduces the gastric injury determined by aspirin. Starting from these data, our study focused on providing multifunctional codrugs obtained joining ASA to tocopherols to take advantage of the synergistic mechanisms regarding the antithrombotic activity of both compounds. In addition, we exploited the protecting role of Vitamin E against gastric side effects of ASA. In the present paper, we reported the synthesis and the *in vitro* hydrolysis of two codrug esters of ASA with α- and γ-tocopherols. We also investigated the oral pharmacokinetic in rabbit and probed the preliminary pharmacological evaluation in rat. The synthesized codrugs exhibited anti-inflammatory activity with a strong and significant reduction in gastrolesivity.

Keyword: *Acetylsalicylic acid, antinflammatory activity, codrug, gastrolesivity, γ-tocopherol, rabbit, rat.*

INTRODUCTION

Acetylsalicylic acid (ASA) is the most widely used drug for the secondary prevention of thrombotic risk in the heart, brain and peripheral circulation [1]. In the brain, aspirin decreases the incidence of thrombotic events due principally to its antiplatelet action [2], which prevents the formation of arterial thrombi. Recently, it has been demonstrated the ability of aspirin to prevent cell damage after a hypoxia by inhibiting oxidative stress, prostaglandin accumulation and inducible nitric oxide synthesis [3,4].

Vitamin E (VE) is a natural antioxidant protecting unsaturated fatty acids, DNA and protein from oxidation. Alpha-tocopherol decreases neuronal cell death caused by ischemia and reperfusion in rat models. [5, 6]. In addition, it has been also demonstrated that alpha-tocopherol influences in vitro the antiplatelet effect of aspirin, mainly by an increase in the prostacyclin and nitric oxide production [7].

Unfortunately, ASA causes gastrointestinal side effects, partly due to its irreversible inhibition of the COX-1-mediated formation of gastric prostaglandin E_2 ($PGE_{2)}$, which is essential for mucosal cytoprotection [8, 9].

Moreover, the stomach damage determined by aspirin seems to involve oxidative stress [10,11]. For this reasons, the co-administration of vitamin E to ASA has been proposed to alleviate the aspirin gastric side effect.

Continuing our interest on vitamin E codrugs [12], the present research focused on providing multifunctional codrugs obtained joining ASA to tocopherols to take advantage of the synergistic mechanism regarding the antithrombotic activity of both compounds. In addition, we exploited the protecting role of VE against the side effects of ASA.

We focused our attention on γ tocopherol for its potent antioxidant properties and anti-inflammatory activity as reported. In fact, γ-tocopherol, but not α-tocopherol, inhibits the proinflammatory eicosanoids, suppresses proinflammatory cytokines, and attenuates inflammation-mediated damage in rat inflammation models [13-14].

Moreover, the esterification of phenolic group on chroman ring of tocopherols is expected to increase the stability to oxidation of the vitamin.

In the present paper, we reported the synthesis and the *in vitro* hydrolysis of two codrug esters of ASA with α- and γ-

tocopherols (Fig. 1). We also investigated the oral pharmacokinetic in rabbit and probed the preliminary pharmacological evaluation in rat. The synthesized codrugs exhibited anti-inflammatory activity with a strong and significant reduction in gastrolesivity.

MATERIALS AND METHODS
Chemistry
NMR spectra were taken on a Bruker AC-250 spectrometer operating at 250.13 (^{1}H) MHz using $CDCl_3$ solutions with TMS as internal standard. Electron impact (EI–MS) mass spectra were recorded on a Kratos MS 50 instrument. HPLC separations were performed on a HP 1100 chromatographic system (Agilent Technologies, Milan, Italy) equipped with a HP ChemStation software, a binary pump G1312A, a diode array detector (DAD) G1315A and a thermostated column compartment G1316A. Elemental analyses (C, H, N) were determined on an elemental analyser Carlo Erba Model 1106 (Carlo Erba, Milano, Italy), and were within 0.4% of the theoretical values. Log D was calculated using ACD/Labs software (version 11.1 2008, ACD, Toronto, ON, Canada). Thin-layer chromatography (TLC) was carried out on Merck Silica Gel 60 F_{254} (0.25 mm thickness). Column chromatography was performed by the flash procedure. Unless otherwise noted, solvents and chemicals (reagent grade or better) were obtained from Sigma-Aldrich (Milano, Italy) and used without further preparation.

Biology
Female New Zealand albino rabbits (Charles River, Calco, Italy), 1.7-2.1 Kg, free of any signs of abnormality were used in the bioavailability study. Adult male rats of the Sprague-Dawley strain (Charles River, Calco, Italy) weighing 170–210 g were used in the pharmacological evaluation. The PGE_2 levels in the inflammatory exudates were analyzed using the Prostaglandin E2 Direct Biotrak Assay (GE Healthcare, Milano, Italy), according to instructions from the manufacturer and as previously described [15].

General procedure for the synthesis of Acetylsalicylic codrugs.
Esters Synthesis
ASA 1.88 g (10.44 mmol) was added of 2.58 g (12.52 mmol) dicyclohexylcarbodiimide in 15 ml of acetonitrile. The resulting suspension was vigorously stirred for 20 min at room temperature. Subsequently, was added a solution of the appropriate tocopherol (6.96 mmol) in 12 ml acetonitrile containing 18 ml of freshly distilled triethylamine. The mixture was left stirring in the dark for 48 at room temperature. The reaction mixture was then filtered and the filtrate was evaporated in vacuo, and the residue was taken up with ethylacetate. The organic phase was then extracted with saturated sodium bicarbonate solution and water. After drying with anhydrous sodium sulfate the solvent was distilled in vacuo and a yellow oil was obtained for both tocopherol derivatives. Thin layer chromatography analysis (hexane:dichloromethane 6:4) shows two intense spots with Rf value of 0.24-0.25 and 0.33-0.34 due to tocopherol esters and the relative unreacted tocopherols, respectively.
Esters Purification
The obtained crude oil ($\approx$ 5.10 g) was mixed with 100 ml of a solution of 50 % $FeCl_3 \cdot 6H_2O$ in methanol and stirred for 1 h at room temperature. After the disappearance of the tocopherol spot (checking by TLC in hexane:dichloromethane 6:4) 300 ml of H_2O were added and the resulting hydroalcholic solution was extracted with dichloromethane (3 x 100 ml). The dichloromethane solution was then extracted with water (3 x 100 ml) and, after drying with anhydrous sodium sulfate, the solvent was distilled in vacuo yielding a yellow oil for both tocopherol ester derivatives ($\approx$ 5.50 g). The obtained oil was purified by flash chromatography using a gradient of hexane:dichloromethane from 6:4 to 4:6.

(2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydro-2H-chromen-6-yl 2-(acetyloxy)benzoate. (VE-α-ASA) (**1**). Isolated as waxy solid. Yield 51%. ^{1}H-NMR δ ($CDCl_3$): δ 8.30 (dd, J_{orto}=7.8, J_{meta}=1.7 Hz, 1H), 7.65 (dt, J_{orto}=7.8, J_{meta}=1.7 Hz, 1H), 7.40 (dt, J_{orto}=7.8, J_{meta}=1.2

Hz, H), 7.19 (dd, J_{orto}=7.8, J_{meta}=1.2 Hz, 1H), 2.62 (t, J=6.8 Hz, 2H), 2.29 (s, 3H), 2.11 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H), 1.79 (m, 2H), 1.55 (m, 3H), 1.25 (s, 3H), 1.48-0.97 (m, 18H), 0.87 (d, J=6.6 Hz, 9H), 0.85 (d, J=6.2 Hz, 3H). MS m/z (rel. int. %): (M^+) 592 (6.7). *Anal.* Calcd for $C_{38}H_{56}O_5$: C, 76.99; H, 9.52; O, 13.49, Found: C, 77.14; H, 9.67; O, 9.64.

(2*R*)-2,7,8-trimethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-chromen-6-yl 2-(acetyloxy)benzoate (VE-γ-ASA) (**2**). Isolated as waxy solid. Yield 52%. ¹H-NMR δ (CDCl₃): δ 8.32 (dd, J_{orto}=7.8, J_{meta}=1.7 Hz, 1H), 7.68 (dt, J_{orto}=7.8, J_{meta}=1.7 Hz, 1H), 7.43 (dt, J_{orto}=7.8, J_{meta}=1.2 Hz, 1H), 7.21 (dd, J_{orto}=7.8, J_{meta}=1.2 Hz, 1H), 6.75 (s, 1H), 2.74 (t, J=6.9 Hz 2H), 2.31 (s, 3H), 2.14 (s, 3H), 2.10 (s, 3H), 1.85 (m, 2H), 1.56 (m, 3H), 1.27 (s, 3H), 1.51-0.99 (m, 18H), 0.89 (d, J=6.6 Hz, 9H), 0.88 (d, J=6.2 Hz, 3H). MS m/z (rel. int. %): (M^+) 578 (6.5). *Anal.* Calcd for $C_{37}H_{54}O_5$: C, 76.78; H, 9.40; O, 13.82; Found: C, 76.98; H, 9.52; O, 13.93.

Hydrolysis studies
The chemical hydrolysis of the synthesized codrugs (20 mg/ml) was studied at 37±0.1° C in buffer phosphate solution at pH 8.0 containing 50% of 2-hydroxypropyl-β-cyclodextrin **(**HP-β-CD). The cyclodextrin was necessary because the low water solubility of the codrugs. At appropriate time intervals samples were taken, diluted 1:10 (v/v) with methanol, filtered and analyzed by HPLC for   acid (SA), α- or γ-tocopherol and the remaining ester codrug.
The synthesized codrugs and the corresponding tocopherols in hydrolysis studies were determined as previously described [12]. Briefly, the separations were obtained with a reverse phase column C18 column (Hypersil ODS; 5 μm, 150 mmx 4.6 mm i.d., obtained from Alltech, Milano Italy) equipped with a direct-connect guard column (Hypersil ODS; 5 μm, 10 mmx 4.6 mm i.d., obtained from Alltech, Milano Italy) using a mobile phase of methanol 100% at a flow rate 2 ml/min. Detection was carried out at 273 nm and external standardization was used. SA was determined using a literature method

[16]. Results of chemical hydrolysis are reported in Fig. 2 and Table I. Data are expressed as means ± SEM.

Bioavailability in rabbit
Rabbits were individually housed in cages in an air conditioned room and maintained on a standard laboratory diet for about 2 weeks before the experiments. The rabbits were kept without food 12 h before the experiments with free access to water. Fifteen minutes before oral administration the rabbits were cannulated via central ear artery using polyethylene tubing. Oral bioavailability was evaluated in three groups of six rabbits. Freshly prepared solutions of test compounds in 5% Tween 80, (VE-α-ASA, VE-γ-ASA, and ASA) were orally administered in separate groups of animals at 10 mg/kg (ASA molar equivalents) by a gastric gavage. An additional group of rabbits was treated with vehicle. At preset interval times 3 ml of blood samples were collected into heparinized tubs for all the groups and immediately analyzed. The volume of blood aspirated was replenished with saline through an intravenous drip during the experiments. For the oral bioavailability study the SA concentration vs time profile was characterized with several different parameters. The maximum drug concentration (C_{max}) and the time at which was drawn (t_{max}) were obtained directly from individual concentration time data. The elimination rate constant (k_{el}) was estimated by least square regression of concentration-time data and half-live was calculated. Finally the area under curves (AUC) from 0 to 24 h was calculated using the trapezoidal rule [17].

Determination of Salicylic Acid plasma concentration
The plasma concentration of SA was determined by a literature method [16]. Fig. 3 shows the time course of plasma SA levels during the bioavailability study. No interfering peaks were observed in the blank plasma chromatogram. Results are expressed as the mean ± SEM.

Carrageenan paw inflammation
Male Sprague–Dawley rats, six for each group, were fasted overnight with access to

water *ad libitum.* Freshly prepared solution of test compounds, VE-α-ASA, VE-γ-ASA and ASA in 5% Tween 80 were orally administered to each group at 150 mg/kg (ASA molar equivalents) by oral gavage 1 h prior to the carrageenan injection (50 μl 1% carrageenan in saline) into the sub plantar region of the right hind-paw pad. An additional group of rabbits was treated with vehicle. Paw volume was determined using the water displacement method [18] as the average of two measurements differing by less than 0.2 ml. Paw volume were assessed 1–4 h post-dosing. Results (Fig. 4) were calculated as the % inhibition of the change in paw volume produced by the carrageenan injection and were expressed as mean ± SEM.

Carrageenan air pouch model

An air pouch was created by a subcutaneous injection of 20 ml of sterile air into the intrascapular area of male Sprague–Dawley rats, six for each group, on days -6 and -3 [19]. On day 0, freshly prepared solutions of test compounds, VE-α-ASA, VE-γ-ASA and ASA in 5% Tween 80 were orally administered at 20 mg/kg (ASA molar equivalents), 1 h prior to the zymosan injection (1.0 ml of a 1% solution) into the pouch. An additional group of rabbits was treated with vehicle. After 4 h, the inflammatory exudates were collected from the pouch for determination of PGE_2 levels by immunoassay. All data are expressed as means ± SEM (Fig. 5).

Gastric injury model

Male Sprague–Dawley rats, six for each group, were fasted overnight with access to water *ad libitum.* Freshly prepared solutions of test compounds, VE-α-ASA, VE-γ-ASA and ASA in 5% Tween 80 were orally administered in separate groups of animals at 250 mg/kg (ASA molar equivalents). An additional group of rabbits was treated with vehicle. Rats were killed 6 h postdosing and their stomachs were removed. The stomachs were examined for macroscopically visible mucosa damage by an observer unaware of the treatment. This involved measuring the lengths (in mm) of all hemorrhagic lesions and calculating a gastric damage score, which

was the sum of the lengths of all lesions in a stomach [20]. Data were expressed as means ± SEM (Fig. 6).

Statistical Analysis

Statistical differences of in vivo data were determined using repeated measure analysis of variance (ANOVA) followed by the Bonferroni-Dunn post hoc pair-wise comparison procedure. A probability, p, of less than 0.05 was considered significant in this study.

RESULTS AND DISCUSSION

Two codrug esters of ASA with α- and γ-tocopherol (1– 2) were prepared using standard carbodiimide procedure and characterized by MS and ^{1}H NMR as well as by elemental analysis. The purification of both codrugs required an additional step because the unreacted tocopherols had Rf values very close to the respective esters (0.33-0.34 and 0.24-0.25, respectively). In addition, both in TLC and in flash column chromatography the ester codrugs and the relative tocopherols showed an intense tailing. In order to improve the chromatographic separations we decided to oxidize the unreacted tocopherols to the respective tocopherylquinones, since, in the elution mixture used, these latter compounds showed higher Rf values with respect to the parent compounds (0.65-0.66).

The oxidation step was easily obtained by treatment of the mixture tocopherol/ester codrug crude oil with a solution of 50 % $FeCl_3 \cdot 6H_2O$ in methanol for 1 h at room temperature. After this procedure the yield of flash chromatographic separation was significantly improved.

Table I. Kinetic data for the hydrolysis of VE-α-ASA and VE-γ-ASA.

|	*VE-α-ASA*	*VE-γ-ASA*
k_{el} (h^{-1})	0.10	0.10
t½ (h)	7.15	7.16

(1)

(2)
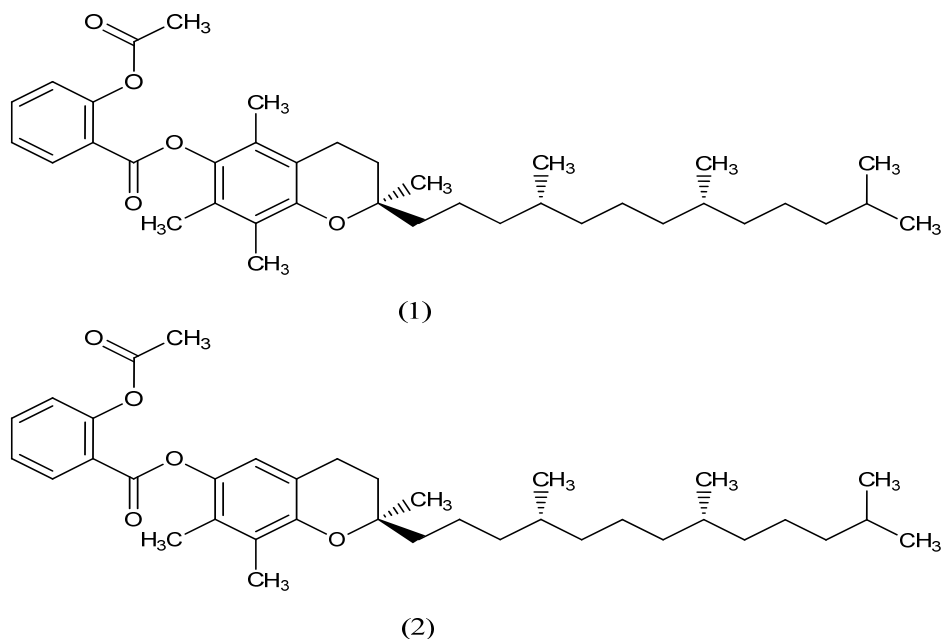
Fig. 1. (2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydro-2H-chromen-6-yl 2-(acetyloxy)benzoate (VE-α-ASA) **(1)** and (2R)-2,7,8-trimethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydro-2H-chromen-6-yl 2-(acetyloxy)benzoate (VE-γ-ASA) **(2)**.

Table II. Pharmacokinetic data of SA released in rabbits treated with ASA, VE-α-ASA and VE-γ-ASA.

| Compound	C_{max} ($\mu g/nl$)	T_{max} (h)	$t\frac{1}{2}$ (h)	k_{el} (h^{-1})	AUC ($\mu g\ ml^{-1}\ h$)	Log D (pH=8.0)
ASA	22.90	1.50	2.20	0.31	98.40±11.60	-1.73
VE-α-ASA	11.22	1.50	7.61	0.09	82.60±9.11	12.11
VE-γ-ASA	11.49	1.50	7.63	0.09	83.86±8.23	11.90

It was expected that VE-α-ASA, VE-γ-ASA ester codrugs would be hydrolyzed in vivo before, during or after absorption to release SA. The relative hydrolysis susceptibility of these compounds were studied in a simulated intestinal fluid at pH 8.0. The VE-α-ASA, VE-γ-ASA ester codrugs were found to be hydrolyzed to SA in chemical hydrolysis studies as revealed by HPLC analysis. The time course of the codrug esters and SA, together with the relative kinetic parameters are shown in table I and Fig. 2. The progress of hydrolysis followed a pseudo first-order kinetics over several half-lives. Examination of data in table I shows that the half-lives at pH 8.0 was 7.15 h and 7.16 h for VE-α-ASA, VE-γ-ASA respectively. These results suggest that the ester codrugs would be hydrolyzed to release SA following oral administration in animal and man. This assumption is well confirmed by the bioavailability study performed in rabbit.
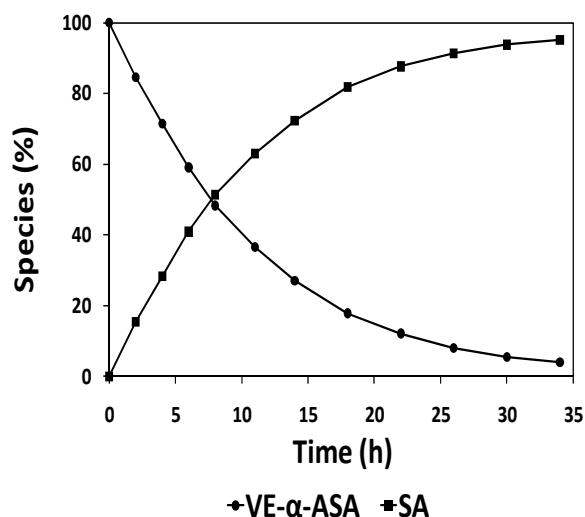
**Fig. 2.** Time course for SA and VE-α-ASA during the hydrolysis of the codrug at pH 8.0.
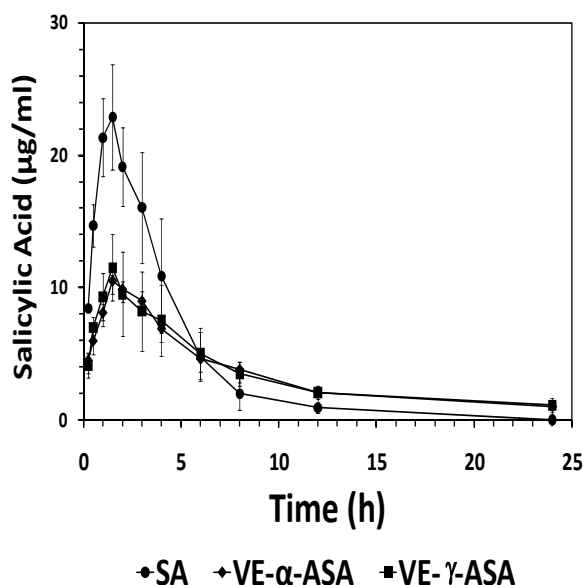
Fig. 3. Salicylic acid (SA) plasma concentration released after treatment of rabbits with VE-α-ASA, VE-γ-ASA and an equivalent dose of ASA (10 mg/Kg).

The time course of SA in rabbits are reported in Fig. 3 and the pharmacokinetic parameters are shown in table II together with the Log D values at pH 8.0. The plasma concentrations of SA, released from ester codrugs, in the time interval 0-6 h were significantly lower (p<0.05) than those derived from ASA. C_{max} values of ester codrugs were lower with respect to ASA with a 2.04 and 1.99 fold decrease for VE-α-ASA and VE-γ-ASA respectively. In the time interval 8-24h the concentrations of SA released by the ester codrugs were higher, but not statistically significant, with respect to ASA. Therefore, the apparent rates of plasma clearance for ester codrugs were slower than that observed with ASA, as demonstrated by the increase in the t½ (from 2.20 h to 7.61-7.63 h) and by the decrease in the K_{el} (from 0.31 to 0.09). The bioavailability measured by the area under the curve was significantly higher for ASA (p<0.05) with an increase of about 15% with respect to the codrugs. No significant differences were registered between the two esters codrugs in all the pharmacokinetic studies.

The overall results suggest that the oral absorption of VE-α-ASA and VE-γ-ASA esters ended later than the ASA due to the more lipophilic character of the codrugs. Probably the increased logD value (Tab. II) of the ester codrugs prolonged their absorption [17].

The anti-inflammatory activity was determined by the carrageenan-induced paw oedema method in rats, at a dose of 150 mg/kg body weight, and compared with the ASA (Fig. 4). VE-α-ASA and VE-γ-ASA showed anti-inflammatory activity lower than ASA during all the experiments. At 1 hour the percent of inhibition were 4.9-5.5 fold lower than ASA for VE-α-ASA and VE-γ-ASA respectively. At 4 hours the differences in the activities were reduced to 1.8-1.9 fold for the codrugs. This trend is in agreement with the pharmacokinetic data (table II). In fact, at 4 hour SA plasma levels ensured by ASA were significantly lower with respect to that registered to the T_{max} by 2.10 fold. At 6 hour we recorded SA plasma levels almost identical for the group treated with ASA and the ester codrugs. There are no significant differences in the activity between the two ester codrugs.
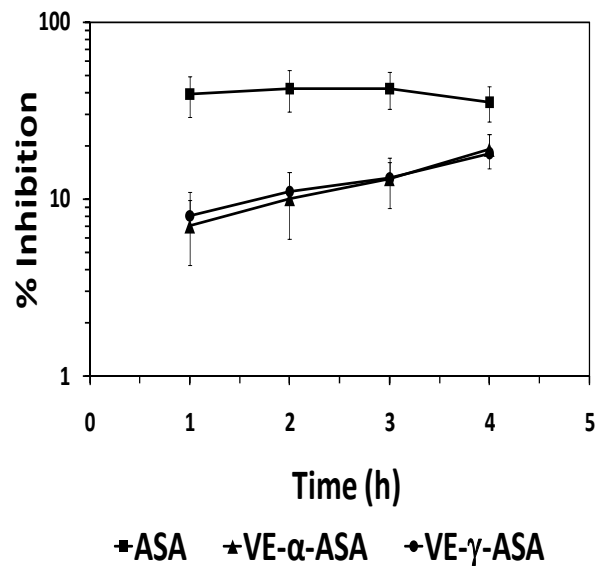

Fig. 4. Inhibition of carageenan-induced paw edema in the rat following oral administration of equimolar doses ASA (150 mg/Kg), VE-α-ASA and VE-γ-ASA.

In the air pouch model ASA and the ester codrugs produced a significant (p<0.05) reduction in the PGE_2 production into the pouch compared to the vehicle control group.

ASA produced a 55% reduction in PGE_2 levels, whereas VE-α-ASA and VE-γ-ASA produced a 22% and 28% decrease in PGE_2 levels respectively. VE-γ-ASA determined a slight higher but not significant reduction (5%) in the PGE_2 production with respect to VE-α-ASA.
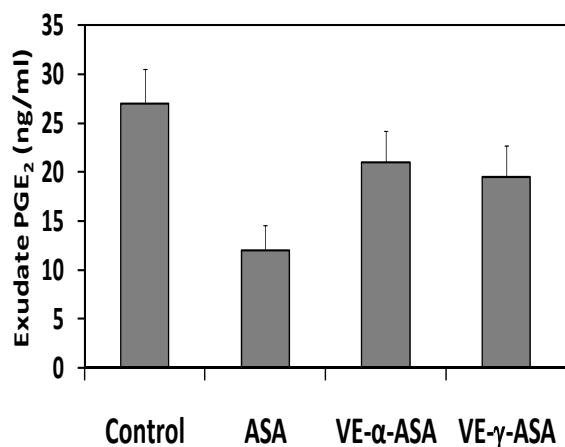
Fig. 5. PGE_2 levels in the inflammatory response in the rat carageenan air pouch model oedema following oral administration of equimolar doses ASA, VE-α-ASA and VE-γ-ASA (20 mg/Kg).
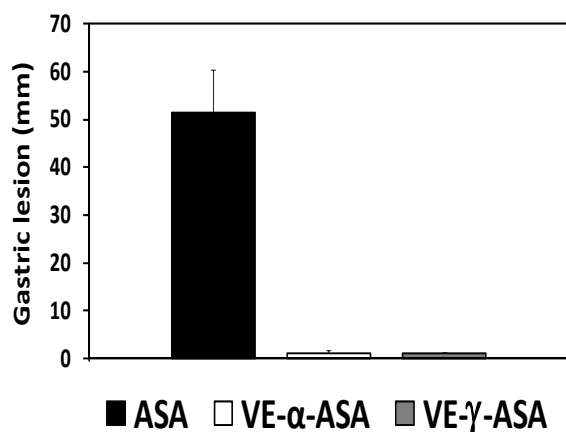
Fig. 6. Gastric lesions scores in the rat following oral administration of equimolar doses (250 mg/kg) of ASA, VE-α-ASA and VE-γ-ASA.

In the gastric injury model (Fig. 5) ASA produced a marked increase in gastric lesions. In contrast, there was a strong and significant (p<0.05) reduction in gastric lesion formation in both VE-α-ASA and VE-γ-ASA treated animals that exhibited almost no lesions.

There was no differences in the gastric lesions in the group of animals treated with VE-α-ASA and VE-γ-ASA.

CONCLUSIONS

Aspirin at low doses is currently recommended for the prophylaxis of cardiac and cerebral ischemic events and is given to almost all patients with coronary artery disease because of its antithrombotic effect [21]. However, the long-term use of aspirin has been prevented by their adverse gastrointestinal effects [21, 22].

Although VE-α-ASA and VE-γ-ASA showed an anti-inflammatory activity lower than ASA in all the pharmacological experiments performed in this study, they were able to significantly reduce PGE_2 production in air pouch model and, at the same time, were completely devoted of gastric side effects. Our preliminary results suggest that VE-α-ASA and VE-γ-ASA, because of their protective gastric action, are promising antinflammatory agents potentially useful for chronic treatment, like in the prevention of thrombotic conditions. Further investigation to better understand antithrombotic potential of these compounds are needed.

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