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Design, Synthesis and Antiinflammatory Activity of Novel γ-Tocopherol Naproxen Ester Prodrug.

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ABSTRACT

Naproxen is widely used for the treatment of arthritic pain. It can induce gastrointestinal (GI) side effects ranging from stomach irritation to ulceration and bleeding. These complications are believed to be determined from the combined effect of the blockage of prostaglandin biosynthesis in the GI tract and direct action of free carboxylic groups. Prodrugs approach have been proposed to overcome the Non-steroidal anti-inflammatory drugs (NSAIDs) side effects by masking the carboxylic acid groups via formation of bioreversible bonds. It has been demonstrated that the production of reactive oxygen species (ROS) plays an important pathogenic role in gastrointestinal ulceration. Our goal was to develop a new Naproxen prodrug which integrates the concepts of prodrug and the beneficial antioxidant effect. For this purpose we designed and synthesized naproxen esters containing tocopherols. Among the possible antioxidant compounds we focused our attention on γ-tocopherol for its potent antioxidant properties and anti-inflammatory activity. In addition, esterification of phenolic group on chroman ring of tocopherols, to form the prodrug, is expected to increase the stability to oxidation of the vitamin.

In the present paper, we report the synthesis and the *in vitro* enzymatic and non-enzymatic hydrolysis of two prodrug esters of naproxen with α- and γ-tocopherols. We also investigated the oral pharmacokinetic in rabbit and probed the preliminary pharmacological evaluation in rat. The synthesized prodrugs exhibited anti-inflammatory activity with a strong and significant reduction in gastrolesivity.

KEYWORDS: Naproxen, γ-tocopherol, prodrug, antinflammatory activity, gastrolesivity, pharmacokinetic, rabbit, rat.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of pain, inflammation and fever. Naproxen (Aleve™, Naprosyn™, Anaprox™, and Naprelan™) is one of the most used NSAIDs for the treatment of arthritic pain. It can induce gastrointestinal (GI) side effects ranging from stomach irritation to ulceration and bleeding [1–3]. These GI complications are believed to be determined from the mixed effect of irritation caused by blockage of prostaglandin biosynthesis in the GI tract and direct action of free carboxylic groups in NSAIDs [4].

The recent failure of the selective COX-2 inhibitors leave the compelling need for effective NSAIDs with improved safety [5].

A variety of prodrugs have been proposed to overcome the NSAIDs side effects and to improve their delivery characteristics by masking the carboxylic acid groups via formation of bioreversible bonds [6-8].

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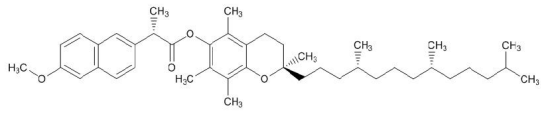
It has been demonstrated that the production of reactive oxygen species (ROS) is increased in NSAIDs therapy. The consequent oxidative damage has been considered to play an important pathogenic role in gastrointestinal ulceration [9-11]. In addition, it has been shown that the combination of antioxidant and anti-inflammatory activity could be helpful in the treatment of a variety of inflammatory conditions by reducing ROS related side effects [12-15].

Our goal was to develop a new NSAID prodrug which integrates the concepts of prodrug and the beneficial antioxidant effect thus we designed and synthesized naproxen esters containing tocopherols.

Among the possible antioxidant compounds 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 [16-17].

It is well known that tocopherols possess a remarkable chemical instability due to the rapid oxidation by atmospheric oxygen. However, esterification of phenolic group on chroman ring of tocopherols to form the prodrug is expected to increase the stability to oxidation.

In the present paper, we report the synthesis and the *in vitro* enzymatic and non-enzymatic hydrolysis of two prodrug esters of naproxen 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 prodrugs exhibited anti-inflammatory activity with a strong and significant reduction in gastrolesivity.



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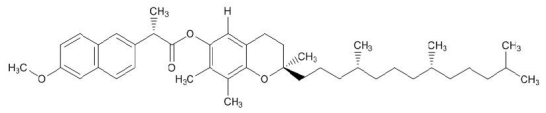

(2)

Fig.1.(2*R*)-2,5,7,8-tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-chromen-6-yl (2*S*)-2-(6-me-thoxynaphthalen-2-yl)propanoate (VE-α-NPX) (1) and (2*R*)-2,7,8-trimethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihycro-2*H*-chromen-6-yl (2*S*)-2-(6-methoxy-naphthalen-2-yl)propanoate (VE-γ-NPX) (2).

EXPERIMENTAL

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), 1.8-2.2 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 180–200 g were used in the pharmacological evaluation. The prostaglandin E2 (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 [18].

General procedure for the synthesis of Naproxen prodrugs.

Naproxen 2.41 g (10.47 mmol) was added of 2.58 g (12.52 mmol) diclohexyl carbodiimmide 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 (40° C, 15mm Hg) 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. The obtained crude oil was then purified by flash chromatography (hexane: dichloromethane 6:4).

(2*R*)-2,5,7,8-tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-chromen-6-yl (2*S*)-2-(6-methoxynaphthalen-2-yl)propanoate (VE-α-NPX) (**1**). Isolated as waxy solid. Yield 66%. ^{1}H-NMR δ ($CDCl_3$): δ 7.82–7.65 (m, 3H), 7.55 (dd, J = 1.7 and 8.5 Hz, 1H), 7.19-7.11 (m, 2H), 4.14 (q, J = 7.1, 1H), 3.90 (s, 3H), 2.53–2.48 (m, 1H), 2.15-0.95 (m, 26H), 2.02 (s, 3H), 1.75 (s, 3H), 1.72 (s, 3H), 1.19 (s, 3H), 0.91–0.75 (m, 12H). MS m/z: 643 (M^+). *Anal.* Calcd for $C_{43}H_{62}O_4$ C, 80.33; H, 9.72; O, 9.95 Found: C, 80.51; H, 9.68; O, 9.93.

(2*R*)-2,7,8-trimethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-chromen-6-yl (2*S*)-2-(6-methoxynaphthalen-2-yl)propanoate (VE-γ-NPX) (**2**). Isolated as waxy solid. Yield 66%. ^{1}H-NMR δ ($CDCl_3$): δ 7.84–7.67 (m, 3H), 7.57 (dd, J = 1.7 and 8.4 Hz, 1H), 7.21-7.13 (m, 2H), 6.52 (s, 1H), 4.17 (q, J = 7.2, 1H), 3.92 (s, 3H), 2.68–2.63 (m, 1H), 2.16-0.97 (m, 26H), 2.05 (s, 3H), 1.81 (s, 3H), 1.21 (s, 3H), 0.92–0.78 (m, 12H). MS m/z: 629 (M^+). *Anal.* Calcd for $C_{42}H_{60}O_4$ C, 80.21; H, 9.62; O, 10.18 Found: C, 80.41; H, 9.72; O, 9.68.

Chemical and Enzymatic Hydrolysis

The chemical hydrolysis of the synthesized prodrugs 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 prodrugs. At appropriate time intervals samples were taken, diluted 1:10 (v/v) with methanol, filtered and analyzed by HPLC for naproxen, α- or γ-tocopherol and the remaining ester prodrug.

The enzymatic hydrolysis was initiated by diluting 1:10 (v/v) appropriate amounts of a stock solution of each prodrug in methanol (1 mg/ml) with a preheated buffer phosphate solution at pH 8.0 containing 5% dimethyl-β-cyclodextrin (DM-β-CD) and porcine liver esterase at 37±0.1° C. The final concentrations in the reaction mixture were 0.15-0.16 μM and 5 IU/ml for the substrates and the enzyme respectively. At appropriate time intervals samples were taken, diluted 1:10 (v/v) with methanol, filtered and analyzed by HPLC for naproxen, α- or γ-tocopherol and the remaining ester prodrug. It has been demonstrated that in this experimental conditions the presence of methanol and DM-β-CD does not influence the enzyme activity [19].

A new HPLC method was developed to simultaneously analyze the synthesized prodrugs and the corresponding tocopherols in hydrolysis studies. 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) was used. Two chromatographic conditions were used with the HPLC apparatus described above. The first, consisting of a mobile phase of methanol 100%, flow rate 2 ml/min and detection at 273 nm for detection of ester prodrugs and relative tocopherols. The second, consisting of a mobile phase of 0.05 M H_3PO_4 (43%) and methanol (57%), flow rate 1 ml/min and detection at 315 nm [20]. Results of chemical and enzymatic hydrolysis are reported in Fig. 2 and Table I. Data are expressed as means ± SEM.

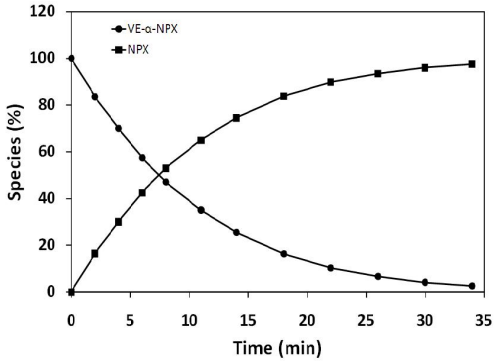

Fig. 2. Time course for Naproxen and VE-α-NPX ester prodrug during the hydrolysis of the prodrug at pH 8.0.

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

	pH 8.0		Enzymatic	
	VE-α-NPX	VE-γ-NPX	VE-α-NPX	VE-γ-NPX
k_{el} (h^{-1})	0.11	0.11	0.31	0.31
$t^{1}/_{2}$ (h)	6.36	6.35	2.27	2.27

Bioavailability in Rabbit

Male rabbits, 2-3 Kg, 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 rabbit 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 male rabbits. Freshly prepared test compounds, VE-α-NPX, VE-γ-NPX, Naproxen or vehicle (5 % Tween 80) were orally administered in separate groups of animals at 7 mg/kg (Naproxen molar equivalents) by a gastric gavage. 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 naproxen 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 [21].

Determination of Naproxen plasma concentration

The plasma concentration of naproxen was determined by HPLC method [22] using indomethacin as an internal standard. 100 µL of plasma was mixed for 5 sec on a vortex mixer with 10 µL of methanol containing 50 µg/ml of indomethacin. The tube was centrifuged at 8,000 rpm for 10 min. An aliquot (10 µL) of the supernatant was injected into the reversed phase column and the flow rate was maintained at 2 ml/min. The mobile phase was 60:40 ratio of methanol and 0.05 M phosphate buffer (pH 5.5). Concentrations of naproxen and indomethacin were detected at 230 nm and quantified by the internal standard method using peak height ratios. Fig. 3 shows the time course of plasma Naproxen levels during the bioavailability study. No interfering peaks were observed in the blank plasma chromatogram. Results are expressed as the mean ± SEM.

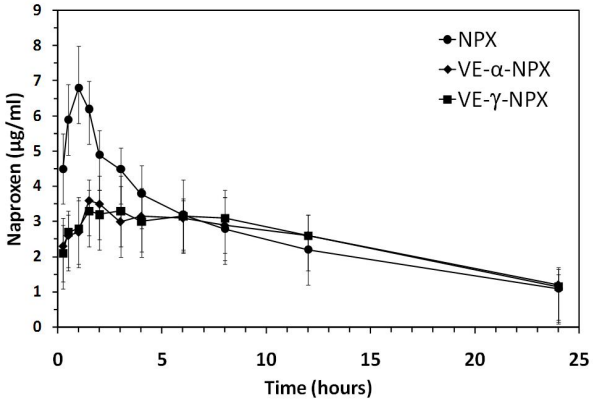

Fig. 3. Naproxen (NPX) plasma concentration after oral administration of VE-α-NPX, VE-γ-NPX and an equivalent dose of NPX.

Carrageenan paw inflammation

Male Sprague–Dawley rats, six for each group, were fasted overnight with access to water *ad libitum.* Freshly prepared test compounds, VE-α-NPX, VE-γ-NPX, Naproxen or vehicle (5 % Tween 80) were orally administered to each group at 10 mg/kg (Naproxen 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. Paw volume was determined using the water displacement method [23] as the average of two measurements differing by less than 0.2 ml. Paw volume were assessed 1–6 h post-dosing. Results (Fig. 4) are calculated as the % inhibition of the change in paw volume produced by the carrageenan injection and are expressed as mean ± SEM.

Carrageenan air pouch model

An air pouch was created by a subcutaneous injection of sterile air into the intrascapular area of male Sprague–Dawley rats, six for each group, on days -6 and -3 [24]. On day 0, freshly prepared test compounds, VE-α-NPX, VE-γ-NPX, Naproxen or vehicle (5 % Tween 80) were orally administered at 5 mg/kg (Naproxen molar equivalents), 1 h prior to the carrageenan injection (1.0 ml of a 1%solution) into the pouch. After 4 h, the inflammatory exudates were collected from the pouch for determination of prostaglandin E2 (PGE_2) levels by immunoassay. All data are expressed as means ± SEM (Fig. 5).

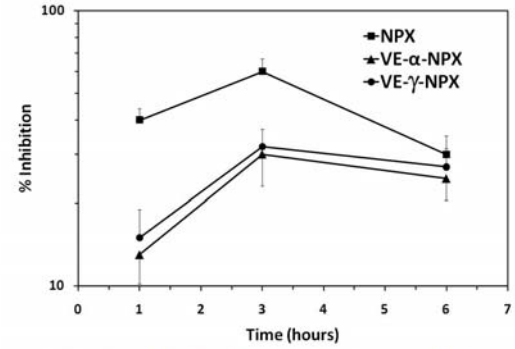

Fig. 4. Inhibition of carageenan-induced paw edema in the rat following oral administration of equimolar doses NPX, VE-α-NPX and VE-γ-NPX (2).

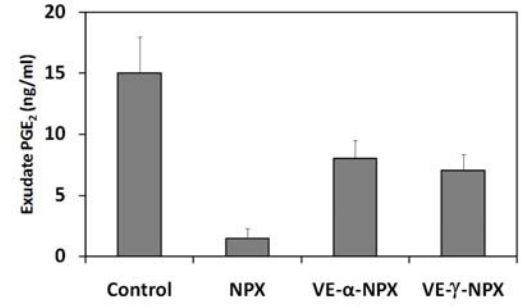

Fig. 5. Prostaflandin E_2 (PGE_2) levels on the inflammatory response in the rat carageenan air pouch model oedema following oral administration of equimolar doses NPX, VE-α-NPX and VE-γ-NPX.

Gastric injury model

Male Sprague–Dawley rats, six for each group, were fasted overnight with access to water ad libitum. Freshly prepared test compounds, VE-α-NPX, VE-γ-NPX, Naproxen or vehicle (5 % Tween 80) were orally administered in separate groups of animals at 10 and 20 mg/kg (Naproxen molar equivalents). Rats were killed 3 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 haemorrhagic lesions and calculating a gastric damage score, which was the sum of the lengths of all lesions in a stomach [25]. Data are expressed as means ± SEM (Fig. 6).

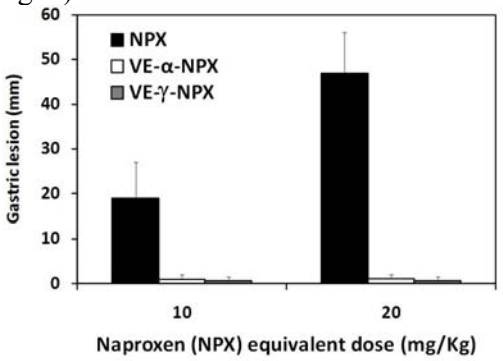

Fig. 6. Gastric lesions scores in the rat following oral administration of equimolar doses (10 and mg/kg) of Naproxen VE-α-NPX and VE-γ-NPX.

Statistical Analysis

Statistical differences of in vivo data are 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 is considered significant in this study.

RESULTS AND DISCUSSION

Two prodrug esters of naproxen with α- and γ-tocopherol (1– 2) were prepared in good yield using standard carbodiimide procedure and characterized by MS and ^1H NMR as well as by elemental analysis. It was predictable that VE-α-NPX, VE-γ-NPX ester prodrugs would be hydrolyzed in vivo before, during or after absorption to release free naproxen which would then

exert its pharmacological action. The relative hydrolysis susceptibility of these compounds were studied in a simulated intestinal fluid at pH 8.0 with and without the presence of hydrolytic enzyme. The VE-α-NPX, VE-γ-NPX ester prodrugs were found to be hydrolyzed to the parent Naproxen in both enzymatic and chemical hydrolysis as revealed by HPLC analysis. The time course of the prodrug esters and Naproxen, together with the kinetic parameters are shown in table I and in Fig. 1. The progress of hydrolysis followed a pseudo first-order kinetics in both chemical and enzymatic trials over several half-lives. Inspection of data in table I shows that the half-lives at pH 8.0 was 6.36 h and 6.35 h for VE-α-NPX, VE-γ-NPX respectively. In presence of porcine esterase the hydrolysis rate was increased of 2.8 fold with a half-lives of 2.27 h for both ester prodrugs. The two prodrugs shows a very similar behavior in both chemical and enzymatic hydrolysis.

These results suggest that the ester prodrugs would be hydrolyzed to release Naproxen following oral administration in animal and man. This speculation is well confirmed by the bioavailability study conducted in rabbit (see below).

The time course of Naproxen plasma level 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 in the time interval 0-6 h following administration of the two ester prodrugs were significantly lower ($p<0.05$) than those for naproxen. For example, the C_{max} values of ester prodrugs were lower with respect to Naproxen with a 1.9 and 2.1 fold decrease for VE-α-NPX, VE-γ-NPX respectively.

In the time interval 8-24h the concentrations of the ester prodrugs were higher, but not statistically significant, with respect to Naproxen. Consequently, the apparent rates of plasma clearance were slower than that observed with naproxen as demonstrated by the increase in the $t_{½}$ (from 9.2 h to 21.0 for the prodrugs) and by the decrease in the K_{el} (from 0.072 to 0.033 for the prodrugs). The bioavailability measured by the area under the curve was significantly higher for Naproxen ($p<0.05$) with an increase of about 11% with respect to the prodrugs. No significant differences were observed between the two esters prodrugs in all the pharmacokinetic study.

Taken together these results suggest that the oral absorption for VE-α-NPX, VE-γ-NPX prodrugs groups ended later than the naproxen group due to the more lipophilic character of the prodrugs. Presumably the logD increased value (Tab. II) of the ester prodrugs prolonged or delayed their absorption during the period in which the plasma concentration of naproxen was falling [21].

The anti-inflammatory activity was determined by the standard carrageenan-induced paw oedema method in rats, at a dose of 10 mg/kg body weight, and compared with the parent naproxen (Fig. 4). VE-α-NPX and VE-γ-NPX showed anti-inflammatory activity lower than naproxen during all the experiment. At 1

Table II. Pharmacokinetic parameters for NPX, VE-α-NPX and VE-γ-NPX in the bioavailability study.

Compound	C_{max} (μg/nl)	T_{max} (h)	$t_{½}$ (h)	k_{el} (h^{-1})	AUC (μg ml^{-1} h	Log D
NPX	6.81	1.00	9.22	0.072	64.18±4.60	-0.18
VE-α-NPX	3.62	1.50	20.99	0.033	57.82±5.11	13.52
VE-γ-NPX	3.38	1.50	21.03	0.033	57.66±5.23	13.34

hour the percent of inhibition were 2.7-3.1 fold lower than naproxen for VE-α-NPX and VE-γ-NPX respectively. At 2 hour all the compounds reached the maximum of the activity and Naproxen was about 1.9-2.0 fold more active than VE-γ-NPX and VE-α-NPX, respectively. At 6 hours the differences in the activities were reduced to 1.1-1.2 fold for the prodrugs 2 and 1 respectively. This trend is in accordance with the pharmacokinetic data. In fact, at 6 hour we recorded naproxen plasma level almost identical for the group treated with Naproxen and the ester prodrugs. Regarding the differences in the activity between the two ester prodrugs we observed that VE-γ-NPX was more active of VE-α-NPX with difference in the range 2-3%. However this differences were not statistically significant.

In the air pouch model, however, there were some differences noted among the three compounds. Naproxen and the ester prodrugs produced a significant ($p<0.05$) reduction in the PGE_2 production into the pouch compared to the vehicle control group. Naproxen produced a 90% reduction in PGE_2 levels, whereas VE-α-NPX and VE-γ-NPX produced a 47% and 53% decrease in PGE_2 levels respectively. VE-γ-NPX determined a slight higher but not significant reduction (6%) in the PGE_2 production with respect to VE-α-NPX.

As shown in Fig. 5, Naproxen produced a dose-dependent increase in gastric lesions. In contrast, there was a strong and significant ($p<0.05$) reduction in gastric lesion formation in both VE-α-NPX and VE-γ-NPX treated animals that exhibited almost absent lesions. There was no differences in the gastric lesions in the group of animals treated with VE-α-NPX and VE-γ-NPX.

In this study the role of γ-tocopherol in the inflammation observed by Jiang [16, 17] was not well demonstrated. In fact, in the preliminary pharmacological evaluation followed in our studies, VE-γ-NPX was slight more active with respect to the VE-α-NPX, but the recorded differences were not statistically significant. This could be due to the insufficient dosage regimen of the tocopherols followed in our experiments. In the Jiang's studies the animals were fed with 100 mg/kg of tocopherols continuously for 3 days. Similar concentrations of tocopherols will require very high dose of ester prodrug that correspond to excessive naproxen doses (until 150 mg/Kg continuously for 3 days).

CONCLUSIONS

NSAIDs are commonly used for pain relief and have consistently been shown to reduce the risk of cancers. However, the long-term use NSAIDs has been hindered by their associated adverse effects, including upper gastrointestinal bleeding and ulcers. Our current study suggests that the ester prodrugs of Naproxen VE-α-NPX and VE-γ-NPX are promising antinflammatory agents in the treatment of chronic inflammatory diseases and inflammation- associated disorders due to protective effect against gastric injury.

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