Journal of Enzyme Inhibition and Medicinal Chemistry, 2009; 24(6): 1351–1356

informa
healthcare

RESEARCH ARTICLE

Trioxsalen derivatives with lipoxygenase inhibitory activity

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Abstract

Trioxsalen (TRX) is a 4,5′,8-trimethylated psoralen analog presenting interesting biological activities when irradiated with UVA light. A series of TRX derivatives, which where obtained by its chemical modification and incorporation of a variety of unsaturated functions at position 4′ of the psoralen ring-system, were evaluated for their antioxidant activity and their inhibitory activity on soybean lipoxygenase (LOX) and lipid peroxidation. The reducing properties of the compounds were evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay and found to be very low, in the range 0–14%, with the exception of the hydroxamic acid **6** which showed almost identical activity to BHT. TRX derivative **3** significantly inhibited LOX, with IC_{50} 9.4 μM. With the exception of TRX, all tested analogs inhibited lipid peroxidation in the range of 35–91%. The most potent compound, namely TRX derivative **3**, was studied for its anti-inflammatory activity *in vivo* on rat paw edema induced by carrageenan, and was found to be of almost identical activity to indomethacin. The results of the biological tests are discussed in terms of structural characteristics.

Keywords: *Psoralens; trioxsalen; trioxsalen derivatives; antioxidant activities; anti-inflammatory activities; lipoxygenase inhibitors; lipid peroxidation*

Introduction

Psoriasis is a chronic inflammatory skin condition. The cause of psoriasis is not known. Several factors are thought to aggravate psoriasis. Japanese researchers believe that they have identified a role for lipoxygenase (LOX) in interferon (IFN)-γ-induced inflammatory processes in psoriasis. Both molecules are known to have inflammatory functions, with LOX involved in the biochemical pathway in which arachidonic acid is metabolized into the proinflammatory products leukotrienes, while IFN-γ has already been implicated in psoriasis[1].

Lipoxygenases play an essential role in the biosynthesis of the leukotrienes (LTs). LTs are potent biological mediators in the pathophysiology of inflammatory diseases and host defense reactions[2]. These properties imply a significant role for LTB_4 in the pathogenesis of inflammatory diseases, such as asthma[3], psoriasis[4], atherosclerosis[5], and cancer[6,7].

Psoralen is the parent compound in a family of natural products known as furocoumarins. Psoralens are naturally occurring or synthetic compounds presenting strong photobiological activities. Psoralens, such as trioxsalen (**TRX**, Figure 1), are commonly used in combination with ultraviolet A (UVA) light for systemic treatment by photochemotherapy (psoralen + UVA = PUVA) of skin hyperproliferative diseases such as psoriasis[8,9]. However, PUVA is associated with important acute and chronic effects[10]. In an effort to obtain psoralens with superior photoreactivity with nucleic acids compared with the parent compound TRX, we have recently synthesized a series of C-4′ psoralen derivatives[11], some of which bear structural characteristics of another well-known antipsoriatic drug, acitretin[10,12]. Since psoralens show cyclooxygenase-2 (COX-2)–5-LOX dual inhibitory activity[13], we considered it interesting to examine the possibility that **TRX** and some

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(Received 19 January 2009; revised 12 March 2009; accepted 25 March 2009)

ISSN 1475-6366 print/ISSN 1475-6374 online © 2009 Informa UK Ltd
DOI: 10.3109/14756360902932776

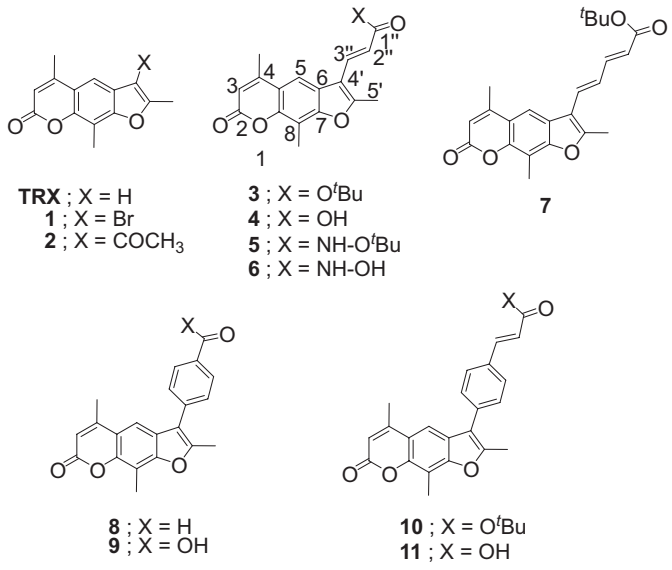

Figure 1. Structures of compounds encountered in the present work.

of the aforementioned derivatives, that is compounds **1–4** and **7–11** (Figure 1), act as inhibitors of lipoxygenase (LOX) activity and of the peroxidation of biological membranes as well as present anti-inflammatory properties. It is worth noting that TRX derivatives such as **4** and **11** incorporate structural characteristics of cinnamic acids. For the sake of completion of the present study, we have now prepared compound **6**, the hydroxamic acid analog of carboxylic acid **4**, and its corresponding *tert*-butyl ester **5**. Through these compounds, we could hopefully determine the effect of the following structural elements: (1) the nature of the substitutent at position 4′ of the psoralen nucleus, (2) the length of the unsaturated side-chain (analogs **3** and **7**), (c) the type of substituent at the end of the unsaturated side-chain (analogs **3–6**), and (d) the type of substituent on position 4 of the phenyl ring, itself attached on the C-4′ of the psoralen nucleus (analogs **8–11**), on the biological activity.

Materials and methods

Materials

Melting points were determined with a Buchi SMP-20 apparatus and are uncorrected. Infrared (IR) spectra were recorded for KBr pellets on a PerkinElmer 16PC Fourier transform (FT)-IR spectrophotometer. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained at 400.13 MHz and ^{13}C NMR spectra at 100.62 MHz on a Bruker DPX spectrometer; tetramethylsilane (TMS) was used as reference. Electrospray ionization mass spectra (ESI-MS) were recorded at 30 V on a Micromass-Platform LC spectrometer using MeOH as solvent. Microanalyses were performed on a Carlo Erba EA 1108 CHNS (carbon, hydrogen, nitrogen, sulfur) elemental analyzer in the Laboratory of Instrumental Analysis of the University of Patras. Flash column chromatography was performed on Merck silica gel 60 (230–400 mesh) and thin layer chromatography (TLC) on Merck 60F_{254} films (0.2 mm) precoated on Al foil. Spots were visualized with UV light at 254 nm and charring agents.

All the chemicals used were of analytical grade and commercially available from Merck. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyl toluene (BHT), and caffeic acid (CA) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Soybean lipoxygenase (type I-B), linoleic acid sodium salt, and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and carrageenan, type K, was commercially available. For the *in vivo* experiments, male and female Fischer-344 rats (180–240 g) were used. **TRX** was purchased from CHEMOS GmbH. The TRX derivatives **1–4** and **7–11** were synthesized according to the methods described in reference 11. The *tert*-butyl ester **5** and hydroxamic acid **6** were obtained as described below.

*(E)-N-tert-butoxy-3-(trioxsalen-4′-yl)acrylamide (**5**).* To an ice-cold suspension of acid **4** (0.60 g, 2 mmol) and HCl.H_2NO^tBu (0.28 g, 2.2 mmol) in anhydrous DMF (4 mL) was added Et_3N (0.96 mL, 6.9 mmol) and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (0.87 g, 2.3 mmol). The resulting reaction mixture was stirred at 0°C for 5 min and at ambient temperature for 15 min and then diluted with $CHCl_3$. The resulting solution was washed sequentially with a 5% aqueous citric acid solution, H_2O, a 5% aqueous $NaHCO_3$ solution, and H_2O, then dried (Na_2SO_4) and finally evaporated to leave a solid residue. The latter was subjected to flash column chromatography using toluene/ethyl acetate (1:1) as the eluent. That way, pure ester **5** was obtained as a white solid [0.61 g (82% yield)]; m.p. 195–198°C; R_f (toluene/ethyl acetate: 1/1) = 0.19; IR (KBr, cm^{-1}): 3270, 1738, 1686, 1664, 1638, 1614, 1598; ^{1}H NMR (d_6-DMSO): δ (ppm) 1.23 (s, 9H, $C(CH_3)_3$), 2.40 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 6.31 (s, 1 H, H-3), 6.58 (d, *J* = 16 Hz, 1H, H-2′′), 7.51 (d, *J* = 16 Hz, 1H, H-3′′), 7.69 (s, 1H, H-5), 10.58 (s, 1H, NH); ^{13}C NMR (d_6-DMSO): δ (ppm) 8.5, 12.9, 19.1, 26.9, 81.4, 108.5, 112.5, 112.9, 113.0, 116.4, 118.7, 122.3, 129.1, 148.9, 153.8, 154.1, 159.5, 160.1, 164.7; ESI-MS (30 eV): *m/z* 761.16 (2*M* + Na), 739.32 (2*M* + H), 392.22 (*M* + Na), 370.07 (*M*H), 314.05 (*M*H-C_4H_8); Anal. Calcd. for $C_{21}H_{23}NO_5$: C, 68.28; H, 6.28; N, 3.79. Found: C, 68.45; H, 6.05; N, 3.62%.

*(E)-N-hydroxy-3-(trioxsalen-4′-yl)acrylamide (**6**).* Ester **5** (0.6 g, 1.6 mmol) was dissolved in ice-cold trifluoroacetic acid (2 mL) and allowed to stand at 0°C for 30 min and at ambient temperature for 1.5 h. Evaporation of the volatile components under reduced pressure left a residue which was triturated with Et_2O and cooled overnight. Pure hydroxamic acid **6** was obtained, as a white solid, by filtration, washing with ice-cold Et_2O, and drying under vacuum (0.43 g, 82%); m.p. 205–208°C (decn.); R_f ($CHCl_3$/MeOH: 9/1) = 0.1; IR (KBr, cm^{-1}): 3448–3270, 3230, 1734, 1718, 1700, 1686, 1676, 1656, 1626, 1598; ^{1}H NMR (d_6-DMSO): δ (ppm) 2.37 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 6.29 (s, 1 H, H-3), 6.51 (d, *J* = 16 Hz, 1H, H-2′′), 7.45 (d, *J* = 16 Hz, 1H, H-3′′), 7.65 (s, 1H, H-5), 9.11 (br. s, 1H, OH), 10.78 (s, 1H, NH); ^{13}C NMR (d_6-DMSO): δ (ppm) 8.9, 13.6, 19.7,

109.2, 111.3, 113.1, 113.4, 114.9, 117.5, 121.8, 139.3, 149.8, 154.7, 155.1, 160.5, 163.3, 164.2; ESI-MS (30 eV): *m/z* 314.95 (*M*H), 282.94 (*M*H-NHOH); Anal. Calcd. for $C_{17}H_{15}NO_5$: C, 65.17; H, 4.83; N, 4.47. Found: C, 65.35; H, 4.67; N, 4.33%.

Biological assays

In vitro *assays*

For the *in vitro* tests a Lambda 20 (PerkinElmer) UV-Vis double beam spectrophotometer was used. Each *in vitro* experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

Determination of reducing activity of stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH)[14]. To an ethanolic solution of DPPH (0.05 mM) in absolute ethanol an equal volume of the compound dissolved in dimethylsulfoxide (DMSO) was added. The mixture was shaken vigorously and allowed to stand for 20 min or 60 min; absorbance at 517 nm was determined spectrophotometrically and the percentage of activity was calculated. All tests were undertaken on three replicates and the results were averaged (Table 1).

Soybean lipoxygenase inhibition study in vitro[14]. The tested compounds dissolved in DMSO were incubated at room temperature with sodium linoleate (0.1 mL) and 0.2 mL of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor.

Inhibition of linoleic acid lipid peroxidation[16]. Production of conjugated diene hydroperoxide by the oxidation of linoleic acid in an aqueous dispersion was monitored at 234 nm. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a free radical initiator. This assay can be used to follow oxidative changes and to understand the contribution of each tested compound.

Azo compounds generating free radicals through spontaneous thermal decomposition are useful for *in vitro* studies of free radical production. The water-soluble azo compound AAPH has been extensively used as a clean and controllable source of thermally produced alkylperoxyl free radicals. Ten microliters of the 16 mM linoleic acid dispersion were added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4, prethermostated at 37°C. The oxidation reaction was initiated at 37°C under air by the addition of 50 μL of 40 mM AAPH solution. Oxidation was carried out in the presence of aliquots (10 μL) in the assay without antioxidant; lipid oxidation was measured in the presence of the same level of DMSO. The rate of oxidation at 37°C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides.

In vivo *assays*

Inhibition of carrageenin-induced edema[14]. Edema was induced in the right hindpaw of Fischer 344-rats (150–200 g) by the intradermal injection of 0.1 mL of 2% carrageenan in water. Both sexes were used. Pregnant females were excluded. Each group was composed of 6–15 animals.

Table 1. Percent interaction with DPPH (reducing activity, DPPH %); *in vitro* inhibition of soybean lipoxygenase (LOX) (IC_{50} or I %); percent inhibition of lipid peroxidation (AAPH %); and percent inhibition of carrageenan-induced rat paw edema (ICPE %).

Compound	Clog *P*[15]	DPPH %, 0.1 mM, 20/60 min	IC_{50} (μM) or I %, LOX, 0.1 mM	AAPH %, 0.1 mM	ICPE %, 0.01 mmol/kg
TRX	3.47	No (20 min) No (60 min)	40%	0	
1	4.33	No (20 min) 100 (60 min)	NA	35	
2	2.98	14 (20 min) 14 (60 min)	79 μM	67	
3	5.03	11 (20 min) 11 (60 min)	9.4 μM	80	46**
4	3.42	11 (20 min) 11 (60 min)	9%	73	
5	5.84	No (20 min) 100 (60 min)	32%	91	
6	3.85	37.5 (20 min) 62 (60 min)	22%	57	
7	6.06	No (20 min) No (60 min)	No	57	
8	4.71	No (20 min) no (60 min)	6%	43	
9	5.10	No (20 min) No (60 min)	No	80	
10	6.92	No (20 min) No (60 min)	33%	41	
11	5.30	No (20 min) No (60 min)	13%	84	
BHT		31 (20 min) No (60 min)			
CA			600 μM		
Trolox				63	
Indomethacin					47**

Note. No, no activity under the reported experimental conditions. The effect on edema is expressed as percent of weight increase of hindpaw (and as percent of inhibition of edema) in comparison to controls. Each value represents the mean obtained from 6 to 15 animals in two independent experiments. In all cases, significant difference from control: **$p < 0.01$ (Student's *t* test).

The animals, which were bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water *ad libitum* during the maintenance period, but they were entirely fasted during the experimental period. Our studies were in accordance with recognized guidelines on animal experimentation. The tested compounds, 0.01 mmol/kg body weight, were diluted in water and were given intraperitoneally, simultaneously with the carrageenan injection. The rats were euthanized 3.5 h after carrageenan injection. Both hindpaws were severed above the ankle joint and were immediately weighed in a very sensitive analytical balance[17]. The difference between the weights of injected and uninjected paws was calculated for each animal. The change in paw weight was compared with that in control animals (treated with water) and expressed as percent inhibition of edema (% ICPE) (Table 1). Indomethacin was tested as a reference compound in 0.01 mmol//kg (47%). Values of % ICPE are the mean from

two different experiments with a standard error of the mean less than 10%.

Biological studies

In vitro *biological studies*

In this investigation all compounds were studied in order to gain insight into their biological response. The C-4′ substituted trioxsalens and the parent molecule **TRX** were studied with regard to their antioxidant ability as well as their inhibition of soybean lipoxygenase.

Results and discussion

In this work, we evaluated *in vitro* and *in vivo* a series of novel TRX derivatives that were expected to offer protection against inflammation and radical attack.

Antioxidants are defined as substances that, even at low concentration, significantly delay or prevent oxidation of easily oxidizable substrates. There has been increased interest in using antioxidants for medical purposes in recent years. It is well known that free radicals play an important role in inflammatory processes[18]. Many non-steroidal anti-inflammatory drugs have been reported to act either as inhibitors of free radical production or as radical scavengers[19]. Consequently, compounds with antioxidant properties could be expected to offer protection in rheumatoid arthritis and inflammation and lead to potentially effective drugs. Thus, we tested the TRX derivatives with regard to their antioxidant ability and in comparison to well known antioxidant agents, e.g. butylated hydroxyl toluene and trolox.

For estimating the antioxidative potential of chemical components, different experimental approaches have been used[20]. Most of them require spectrophotometric measurement and a certain reaction time in order to obtain reproducible results[21]. The DPPH assay is used as a radical scavenging measuring method. DPPH is a stable free radical in methanolic solution. In its oxidized form, the DPPH radical has an absorbance maximum centered at about 517 nm[22]. The DPPH assay is described as a simple, rapid, and convenient method independent of sample polarity for the screening of many samples for radical scavenging activity[23]. These advantages make the DPPH method interesting for testing our analogs.

The interaction of the examined compounds with the stable free radical DPPH is shown in Table 1. This interaction indicates their radical scavenging ability in an iron-free system. Only TRX derivative **6** (37.5%) showed an almost equipotent interaction to the reference compound BHT (31%). Compounds **2–4** presented very low interaction to DPPH at 0.1 mM (11–14%), whereas compound **TRX** and derivatives **1**, **5**, and **7–11** did not show any interaction at 0.1 mM. The interaction remained in most cases highly constant. With the exception of TRX derivative **6** and the TRX derivativatives **1** and **5,** which showed 100% interaction after 60 min, no changes were observed after 60 min for the rest of the derivatives. It is evident that the presence of the hydroxamate function correlated with high interaction values, whereas blocking the free hydroxyl group in ester **5** completely suppressed this interaction. No changes were observed between the acid **4** and its *tert*-butyl ester **3**. The insertion of a double bond in the side-chain as well as a phenyl group did not influence notably the antioxidant activity.

Leukotrienes play an important role as mediators of a variety of inflammatory and allergic reactions and are derived from the biotransformation of arachidonic acid catalyzed by lipoxygenase (LOX). Lipoxygenases play a role in membrane lipid peroxidation by forming hydroperoxides in the lipid bilayer[24]. Inhibitors of LOX have attracted attention initially as potential agents for the treatment of inflammatory and allergic diseases, but their therapeutic potential has now been expanded to certain types of cancer and cardiovascular diseases.

In this context, we decided to further evaluate the synthesized TRX derivatives for inhibition of soybean lipoxygenase (LOX) by the UV absorbance based enzyme assay[25] (Table 1). Most of the LOX inhibitors are antioxidants or free radical scavengers[26], since lipoxygenation occurs via a carbon-centered radical. Some studies suggest a relationship between LOX inhibition and the ability of the inhibitors to reduce Fe^{3+} at the active site to the catalytically inactive Fe^{2+}. LOXs contain a "non-heme" iron per molecule in the enzyme active site as high-spin Fe^{2+} in the native state and high-spin Fe^{3+} in the activated state. Several LOX inhibitors are excellent ligands for Fe^{3+}. This inhibition is related to their ability to reduce the iron species in the active site to the catalytically inactive ferrous form.

Perusal of the IC_{50} or percent inhibition values (Table 1) shows that TRX derivative **3** is the most active (9.4 μM) within the set followed by compound **2** (79 μM). It is therefore apparent that the highest inhibitory activity is secured by the trioxsalen derivative **3**, bearing a highly lipophilic α,β-unsaturated *tert*-butyl ester side-chain, followed by compound **2**, bearing also an unsaturated and lipophilic group, namely the acetyl group. It is also apparent that all esters in this series of compounds presented higher interaction values with the enzyme compared to their corresponding acids. It is also interesting to note that insertion of a second double bond in the side-chain diminished this interaction, whereas a phenyl group had a much less deleterious effect (compounds **3**, **7**, and **10**). Although lipophilicity is referred to[27] as an important physicochemical property for LOX inhibitors, all the above tested derivatives do not follow this concept.

In this investigation, all compounds were studied in order to identify their possible inhibitory activity on lipid peroxidation. Azo compounds generating free radicals through spontaneous thermal decomposition are useful for free radical production studies *in vitro*. The water-soluble azo compound AAPH has been extensively used as a clean and controllable source of thermally produced alkylperoxyl free radicals. In our studies, AAPH was used as a free radical initiator to follow oxidative changes of linoleic acid to conjugated diene hydroperoxide. Compounds **3**, **5**, **9**, and **11** showed higher inhibition (80–91%) of lipid peroxidation

than the reference compound trolox (63%). It is interesting to note that among the analogs with a double bond in the side-chain, the *tert*-butyl esters are more potent inhibitors than the corresponding acids, but this is reversed in the analogs with a phenyl ring in the side-chain, wherein the acids present higher inhibitory activity.

Regression analyses of the values of percent inhibition of lipid peroxidation (AAPH %) for the subgroup of compounds **2–4**, **7**, and **9–11** revealed that low lipophilicity is the main physicochemical parameter influencing their interaction, followed by an indicator variable taking the value 1 for the presence of a free COOH group. A free COOH group is more significant for the inhibition of lipid peroxidation than an ester group:

$$\text{Log(AAPH)}\,\% = -0.154\,(0.073)\,\text{Clog}\,P + 0.315\,(0.206)\,\text{I}_{\text{COOH}} + 2.338\,(0.247)$$

$$n = 7,\ r = 0.948,\ r^2 = 0.898,\ q^2 = 0.574,$$
$$s = 0.043,\ F_{2,4} = 18.57,\ \alpha = 0.01$$

The most potent compound in this set of TRX derivatives, namely ester **3**, was selected to be examined *in vivo* using the functional model of carrageenan-induced rat paw edema, on the basis that it both highly inhibited LOX and presented high *in vitro* antioxidant activities. Carrageenan-induced edema is a non-specific inflammation resulting from a complex of diverse mediators[28]. As shown in Table 1, this compound induced equipotent inhibition, essentially identical to the effect produced by the commonly used standard, namely indomethacin.

Conclusion

A series of TRX derivatives, bearing a variety of substituents at position 4′ of the psoralen ring-system, were synthesized using literature procedures or typical peptide synthetic methods, and their reducing abilities were determined using the stable radical DPPH at 0.1 mM after 20–60 min. The reducing abilities ranged from 0 to 37.5% (20 min) and 0 to 100% (60 min). This interaction expresses the reducing activity of compounds and indicates their ability to scavenge free radicals. The highest activity was shown by TRX derivative **6**, bearing an α,β-unsaturated free hydroxamic acid function as side-chain. Inhibitory activities were measured against soybean lipoxygenase, *in vitro*. The compounds were tested in several concentrations and IC_{50} or percent inhibition values were determined. TRX derivative **3** showed the best inhibitory activity followed by TRX derivative **2**, bearing an α,β-unsaturated *tert*-butyl ester and an acetyl side-chain, respectively. TRX derivative **3** showed much higher inhibition on lipoxygenase compared to caffeic acid and better inhibitory activity of lipid peroxidation compared to trolox. The highest inhibition of lipid peroxidation was shown by the *tert*-butyl ester **5** of the hydroxamic acid **6**, followed by the TRX derivatives **11** and **9**, bearing a benzoic or cinnamic acid side-chain, and compound **3**. Finally, compound **3** with the most promising biological profile also showed equipotent anti-inflammatory activity *in vivo* to indomethacin.

In general, the presence of an α,β-unsaturated hydroxamic acid side-chain at position C-4′ of the psoralen ring-system seems to increase substantially the antioxidant properties of trioxsalen. On the other hand, the highest inhibitory ability of LOX is secured by the presence of *tert*-butyl acrylate or acetyl side-chains, whereas the highest inhibition of lipid peroxidation is shown by derivatives with *tert*-butyl acrylate or α,β-unsaturated hydroxamate side-chains and benzoic or cinnamic acid side-chains. Finally, incorporation of the *tert*-butyl acrylate side-chain at position 4′ of the psoralen ring-system provides not only the strongest inhibitor of LOX and strong inhibition of lipid peroxidation within this family of compounds but also significant anti-inflammatory activity.

Acknowledgements

We thank the European Social Fund (ESF) Operational Programme for Educational and Vocational Training II (EPEAEK II) and particularly the Program PYTHAGORAS II for funding the synthetic part of this work. We would also like to thank Dr. C. Hansch and Biobyte Corp., Claremont, CA, USA for free access to the C-QSAR program.

A part of this research has been presented at the 13th Panhellenic Symposium of Pharmacochemistry, 2008.

***Declaration of interest*:** The authors report no conflicts of interest.

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