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RESEARCH ARTICLE

Targeting Inflammation with Conjugated Cinnamic Amides, Ethers and Esters

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Abstract: ***Background*:** Cinnamic acid is a key intermediate in shikimate and phenylpropanoid pathways. It is found both in free form, and especially in the form of esters in various essential oils, resins and balsams which are very important intermediates in the biosynthetic pathway of several natural products. The cinnamic derivatives play a vital role in the formation of commercially important intermediate molecules which are necessary for the production of different bioactive compounds and drugs. Different substitutions on basic moiety lead to various biological activities. Furthermore, combination of appropriate pharmacophore groups with cinnamic acid derivatives were developed to give hybrids in order to find out promising drug candidates as inhibitors of multiple biological targets associated with inflammation. We found interesting to continue our efforts to design and synthesise three series of novel cinnamic acid-based hybrids: a) nitrooxy esters of cinnamic acid, b) ethers and c) amides of cinnamic acids with arginine, as pleiotropic candidates against multiple targets of inflammation

***Methods*:** The synthesis of cinnamic was established by a Knoevenagel-Doebner condensation of the suitable aldehyde either with malonic acid in the presence of pyridine and piperidine, or with phenylacetic acid in the precence of triethylamine in acetic anhydride. The synthesis of the corresponding esters was conducted in two steps. The ethers were synthesized in low yields, with 1,2 – dibromoethane in dry acetone, in the presence of K_2CO_3, to give oily products. The corresponding cinnamic amides were synthesised in a single step. The synthesised hybrids were tested as lipoxygenase (LOX) and cyclooxygenase (COX) inhibitors *in vitro*. *In silico* docking was applied to all the novel derivatives. Several molecular properties of the hybrids were calculated in order to evaluate their drug likeness.

***Results*:** A number of esters, ethers and amides of selected cinnamic acids, either phenyl substituted or not, has been synthesised and subjected to modelling studies. The compounds were studied *in vitro/in vivo* for their inhibitory activities on cox and lox, and as antioxidants. Log *P* values of all the title compounds except of 3a (5.38) were found to be less than 5 and are in agreement to Lipinski's rule of five, suggesting satisfactory permeability across cell membrane. The molecular modelling study seems to be in accordance with the experimental results for LOX and COX-2. The result of antioxidant activity for amide **3b** supports the anti-lox activity. Compound **5d** presents the higher *in vivo* anti-inflammatory.

***Conclusion*:** According to the experimental findings compounds **3b** and **5d** can be used as lead compounds for the design of new molecules to target inflammation.

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1. INTRODUCTION

Cinnamic acid is a key intermediate in the shikimate and phenylpropanoid pathways. It is found both in free form, and especially in the form of esters (ethyl, cinnamyl, benzyl), and in various essential oils, resins and balsams which are very important intermediates in the biosynthetic pathway of several natural products. The cinnamic derivatives play a vital role in the formation of commercially important intermediate molecules which are necessary for the production of different bioactive compounds and drugs. Cinnamic acid derivatives present a wide range of biological activities: Antituberculosis [1], antidiabetic [2], antioxidant [3] antimicrobial [4], hepatoprotective [5], Central Nervous System Stimulant (CNS) [6], antidepressant [7], anticholesterolemic [8], antimalarial [9], antiviral [10], anxiolytic [11], cytotoxic [12]

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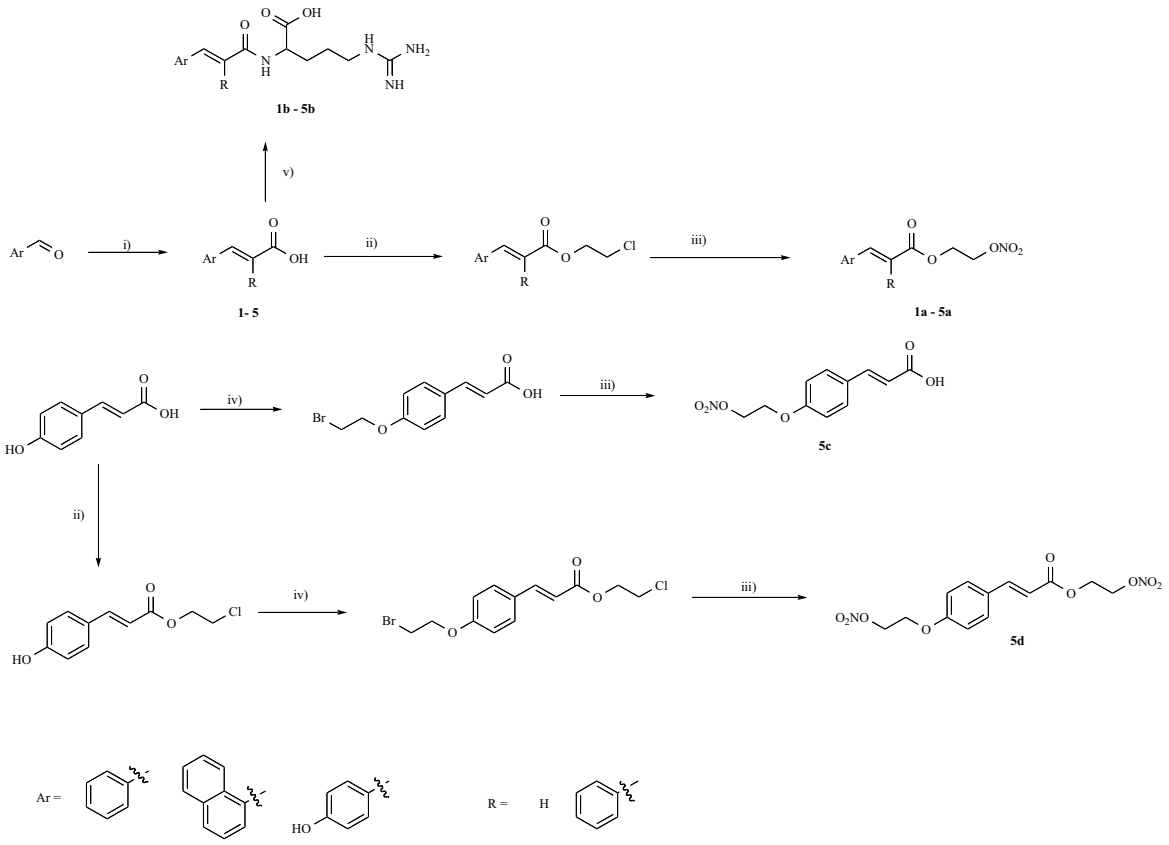
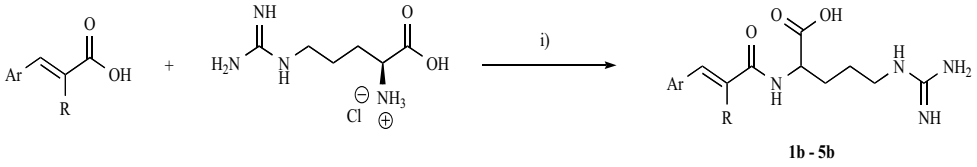
1875-628X/20 $65.00+.00



Scheme (1). Reagents and Conditions: **i**) malonic acid, cat. piperidine, pyridine or phenylacetic acid, cat. TEA, Ac_2O, **ii**) 1 chlorethanol, DCC, cat. DMAP, DCM (dry), **iii**) NH_4NO_3, MeCN, **iv**) 1,2 - dibromoethane, K_2CO_3, Acetone (dry). **v**) L – Arginine hydrochloride, BOP, TEA, DCM (dry).



Scheme (2). Reagents and Conditions: **i**) BOP, TEA, DCM (dry).

and anti-inflammatory [13]. Furthermore, the combination of appropriate pharmacophore groups with suitable substituted cinnamic acids led to conjugates with anti-inflammatory activities [3]. In recent years, intensive research on cinnamic acid derivatives has been conducted in order to create new multifunctional drugs [14, 15]. We designed and synthesised [16-20] several cinnamic acids as potent lipoxy genase (LOX) and cyclooxygenase (COX-2) inhibitors, antioxidants and anti-inflammatories over the last decade. L-arginine is characterized as a semi-essential amino acid influencing the stages of development and health. The infants cannot synthesize L-arginine thus rendering it an essential amino acid. Furthermore, there is a need for arginine in inflammatory cases such as burns, certain surgeries and sepsis [21].

2. MATERIALS AND METHODS

L-Arginine is a precursor of endogenous nitric oxide (NO) [22]. It was also proved that exogenous L-arginine possesses superoxide scavenging activity and is able to delay the cell-mediated breakdown of NO as well as to reduce the oxidation of lipoproteins [23].

Previously reported [17] potent anti-LOX cinnamic acids *e.g.* the cinnamic acid, the *p*-coumaric acid and the naphthyl substituted acid were used for the synthesis of the esters, ethers and amides. In this light, we esterified our cinnamic acids with 2-nitrooxyethanol. Furthermore, we synthesized some ethers of *p*-coumaric acid as pleiotropic candidates against multiple targets of inflammation [24, 25]. These new derivatives can be divided into three categories: **a**) nitrooxy esters of cinnamic acids, **b**) ethers and **c**) amides of cinnamic acids with arginine, and have been evaluated for their: **a**) antioxidant activity [26] **b**) *in vitro* ability to inhibit soybean lipoxygenase [16], **c**) *in vitro* ability to inhibit cyclooxygenase-2 [27] and **d**) *in vivo* anti-inflammatory activity using the carrageenin mice paw edema [28, 29] (Supplementary material).

We used modelling studies and previous QSAR results [30] as a tool in our design for LOX inhibitors. The *in silico* results supported the synthesis of the compounds.

The drug-likeness of the derivatives was determined from the theoretical calculation of various molecular properties *e.g.* partition coefficient (log *P*), Topological Polar Surface Area (TPSA), hydrogen bond donors and acceptors, rotatable bonds, number of atoms, molecular weight. The violations of Lipinski's rule of five were considered [30].

3. RESULTS AND DISCUSSION

The design of the new multitarget cinnamic derivatives was based on our previous research [25] combining the moiety of the enoyl-acyl backbone part, the amide or the ester linkage. (Schemes **1** and **2**). Variations were accomplished by the choice of suitable substituted cinnamic acids which showed interesting biological activity in our previous work [25].

The synthesis of cinnamic acids **1, 2, 3,** and **4** was established by a Knoevenagel-Doebner condensation of the suitable aldehyde either with malonic acid in the presence of pyridine and piperidine, or with phenylacetic acid in the presence of triethylamine in acetic anhydride, as shown in Scheme **1** [25]. The physicochemical and spectroscopic data of the obtained acids are identical to those given in the literature.

The synthesis of the corresponding esters was conducted in two steps. The intermediate 2 chlor ethyl ester [31] of the appropriate acid reacts with ammonium nitrate to give the corresponding alkyl nitro-ester. The ethers were synthesised in low yields, with 1,2 - dibromoethane in dry acetone, in the presence of K_2CO_3, to give oily products. The corresponding cinnamic amides were synthesized in one pot by the reaction of the appropriate acid with L Arginine HCl and (Benzotriazol-1-yloxy) tris (dimethylamino) phosphonium hexafluorophosphate (BOP) as coupling reagent [32] Compound **1b** was synthesized for the first time by our group, while so far in the literature [33] only its isolation from natural sources has been described. The final amide products were obtained in medium to good yields (50-92%).

The crude amides were recrystallized from ethanol and the esters were subjected to Preparative Thin Layer Chromatography (PTLC). IR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis were used for the confirmation of the synthesized compounds' structures. All the esters and amides presented the characteristic absorption in the IR (KBr disk) 1720 (C=O), 1625 (C=C), (cm^{-1}) and correspond to the *E*-isomers (*J*> 9 Hz). The ^{1}H-NMR and ^{13}C-NMR data confirmed the proposed structures and were in agreement with the literature [16-18]. The LC-MS (ESI) analysis showed: $[M+H]^+$ as well as $[M+Na]^+$, $[M+K]^+$, $[M+Na+MeOH]^+$, and the peak at m/z 113 for some amides, as mentioned in the literature [33] (Tables **1**-**2**).

3.1. Physicochemical Studies

We tried to determine the lipophilicity of the synthesized derivatives experimentally, using the RPTLC method as R_M values [24], since lipophilicity is a significant physicochemical property determining the ADME properties. We succeeded to correlate the theoretically calculated lipophilicity values calculated with C-QSAR (as clog *P)* to the R_M values for the esters (Tables **1** and **2**). The following equation describes this correlation:

$R_M = 0.225\ (0.128)\ \text{clog}\ P - 1.492\ (0.511)$

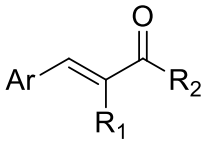
$N = 6,\ r = 0.926,\ r^2 = 0.857,\ q^2 = 0.627,\ s = 0.106,\ F_{1,4} = 24.09,\ \alpha = 0.01$

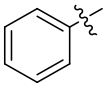
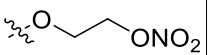
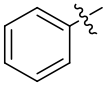
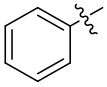
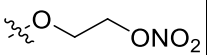
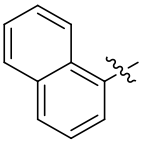
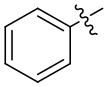
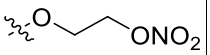
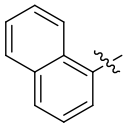
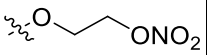
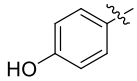
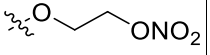
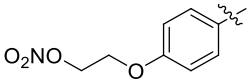
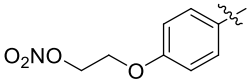
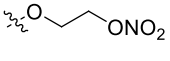
We did not succeed to correlate the corresponding R_M to clog *P* values for the amides. This may be due to the limited number of compounds (**4**) and the different nature of the hydrophilic and lipophilic phases used within the two systems.

Several molecular properties of the derivatives were theoretically calculated in order to evaluate their drug-likeness [34]. All the compounds have a molecular weight less than 500. Thus, these molecules could be easily transported, diffused and absorbed. Counting the number of hydrogen bond acceptors (O and N atoms) and the number of hydrogen bond donors (NH and OH) in the synthesized compounds, it seems that both properties follow the Lipinski's rule of five (less than 10 and 5 respectively). Within the series of the examined derivatives, compounds **1a**-**5a** and **5c** seem to be orally active in accordance to Lipinski's rule of five. The hydrogen bonding of the compounds is highly correlated to the topological polar surface area. This property is used as a significant indicator of the bioavailability of a bioactive molecule. The TPSA of the derivatives was observed in the range of 81.36-148.53 Å and is well below the limit of 160 Å, indicating good oral bioavailability. The upper limit for TPSA for a molecule to penetrate the brain is around 90 Å. The Log *P* values of all the title compounds except of **3a** (5.38) were found to be less than 5 and are in agreement to Lipinski's rule of five, suggesting satisfactory permeability across the cell membrane. The *in silico* predicted values [36], point that compounds with logBB values more than 0.3 are considered to be highly absorbed through BBB whereas values between 0.3 to - 0.1 and less than -0.1 are considered to be limited transported through BBB. Our findings do not support the permeability of these hybrids through BBB (Table **3**).

The synthesized derivatives were expected to offer inhibition of lipoxygenase (LOX), cyclooxygenase (COX-2), protection against radical attack and *in vivo* anti-inflammatory activity. The compounds were tested as lipoxygenase and cyclooxygenase inhibitors *in vitro*. Both enzymes catalyze the arachidonic acid metabolism giving chemical mediators of inflammation. Free radicals are highly implicated with lipoxygenase inhibition and inflammation Thus, we used the water-soluble AAPH which generates *in vitro* free radicals through spontaneous thermal decomposition, in order to study the anti-lipid peroxidation activity of the derivatives. The experimental conditions employed in our study significantly resemble the cellular lipid peroxidation due to the activity of the peroxyl radicals. The compounds were tested at 100μM. With the exception of the esters **2a**, **5a** and **5d** as well as of the amides **1b**, **4b** and **5b**, all the other derivatives showed high anti-lipid peroxidation activity. The presence of a phenyl substituent increases the anti-lipid peroxidation activity of ester **3a** in comparison to **4a**. Ether **5c** seems to be more potent than ester **5a** whereas the ether-ester **5d** was found to be less potent than both **5c**

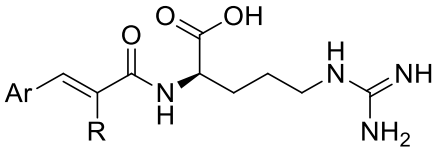
Table 1. Lipophillicity values of cinnamic ester hybrids (clog *P* and Rm values); % inhibition of lipid peroxidation (% LP); % *in vitro* soybean LOX inhibition (%LOX inh.); % *in vitro* ovine COX – 2 inhibition (% COX-2 inh.); % *in vivo* inh. of caraggenin paw edema CPE% (±SD) (0.01 mol/mL/kg body weight).

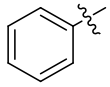
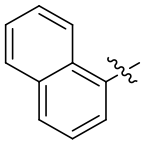
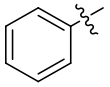
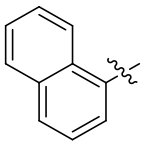
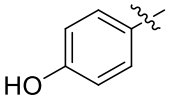


Compounds	Ar	R_1	R_2	Clog P[#]	R_M[##](±SD)	% LP inh@ (100µM)	%LOX inh.@ (100 µM)	%COX-2 inh. @ (100 µM)	CPE% (±SD)(0.01 mol / ml/ kg Body Weight
1a		H		2.75	-0.696 (±0.044)	86.0	41.0	no	36(±0.2)*
2a				4.09	-0.482 (±0.04)	32.0	no	no	n.t.
3a				5.27	-0.289 (±0.03)	74.8	no	no	n.t.
4a		H		3.92	-0.447 (±0.01)	67.4	no	no	27.7 (±0.8) **
5a		H		2.08	-0.751 (±0.015)	no	23.0	no	24(±0.6) **
5c		H	OH	2.44	-0.954 (±0.004)	86.5	9.4	no	n.t.
5d		H		2.95	-0.820 (±0.053)	9.6	no	62.5/ IC_{50} =82.5 µM	55.5 (±3.8)**
NDGA	-	-	-	-	-	-	93 (IC_{50} = 0.55 µM)	-	-
Trolox	-	-	-	-	-	88	-	-	-
Indomethacin	-	-	-	-	-		-	95	37.3 (±1.3)**

[#]clog *P*: theoretically determined values *via* C – QSAR program; [##] R_M values are the average of at least 5 measurements; SD <10%; no: The compounds did not show any activity under the reported experimental conditions; nt not tested; *p<0.01, **p<0.1 compared to reference (Student's T-test).

Table 2. Lipophillicity values of cinnamic amides hybrids (c log *P* and Rm values); % inhibition of lipid peroxidation (% LP); % *in vitro* soybean LOX inhibition (%LOX inh.); % *in vivo* inh. of caraggenin paw edema CPE% (±SD) (0.01 mol/mL/kg body weight).



Compounds	Ar	R	Clog *P**	R_M** (±SD)	% LP inh@ (100µM)	%LOX inh.@ (100 µM)	CPE% (±SD) (0.01 mol/mL Kg Body Weight)
1b		H	-1.53	0.363 (±0.006)	no	33.8	28.4(±0.7)*
3b			0.99	-0.04 (±0.001)	57.9	57.4 (IC_{50} = 51µM)	64.8 (±3.7)*
4b		H	-0.36	0.273 (±0.006)	no	no	42(±1.3)*
5b		H	-2.20	0.398 (±0.001)	0.9	no	nt
NDGA	-	-	-	-	-	93 (IC_{50} = 0.55 µM)	-
trolox	-	-	-	-	88	-	-
indometha-cin	-	-	-	-	-	-	37.3 (±1.3)**

*clog *P*: theoretically determined values *via* C – QSAR programme; ** R_M values are the average of at least 5 measurements; SD <10%; no: the compounds did not show any activity under the reported experimental conditions; *p<0.01, **p<0.1 compared to reference (Student's T-test).

Table 3. Molecular properties prediction-Lipinski "Rule of five". Drug likeness of the synthesised compounds.

Compounds	milogP[a]	TPSA[b]	N° atoms	N° O,N[c]	N° OH, NH[d]	N° violations	N° rotational bonds[e]	Volume[f]	MW[g]	logBB[h]
1a	3.06	81.36	17	6	0	0	7	205.35	237.21	-0.1753
2a	4.40	81.36	23	6	0	0	8	276.76	313.30	0.0324
3a	5.38	81.36	27	6	0	1	8	320.75	363.37	-0.0234
4a	4.04	81.36	21	6	0	0	7	249.34	287.27	0.1843
5a	2.59	101.59	18	7	1	0	7	213.37	253.21	-0.45045
5c	2.60	101.59	18	7	1	0	7	213.37	253.21	-0.4489
1b	-1.05	128.30	22	7	6	1	9	283.07	304.35	-1.28175

(**Table 3) Contd…**

Compounds	milogP[a]	TPSA[b]	N° atoms	N° O,N[c]	N° OH, NH[d]	N° violations	N° rotational bonds[e]	Volume[f]	MW[g]	logBB[h]
3b	1.27	128.30	32	7	6	1	10	398.47	430.51	-0.92215
4b	-0.07	128.30	26	7	6	1	9	327.06	354.41	-1.12985
5b	-1.53	148.53	23	8	7	1	9	291.90	320.35	-1.55845
5d	3.75	145.66	24	11	0	1	12	280.26	342.26	-0.71135

[a]Logarithm of partition coefficient between n-octanol and water (milog *P*); [b]Topological Polar Surface Area (TPSA); [c]Number of hydrogen bond acceptors (n-ON); [d]Number of hydrogen bond donors (n-OHNH); [e]Number of rotatable bonds (n-rotb); [f]Molecular Volume; [g]Molecular Weight; [h] Blood Brain Barrier (BBB).

and **5a**. Within the amide group, **3b** presented higher antioxidant activity than **4b** in which the phenyl substituent was absent.

The performed molecular modelling study provided a useful interpretation of the experimental results [35-43]. The preferred docking pose for the most potent derivative **3b** is shown in Fig. (**1**). The binding of **3b** to soybean LOX (PDB code: 3PZW) has a higher AutoDockVina score (-10.1) compared to the other docked derivatives. From the docking results, it seems that the designed derivatives present allosteric interactions with the enzyme. Furthermore, **3b** is able to accommodate the extensively hydrophobic cavity close to the active site with possible hydrophobic interactions (π-π stacking).

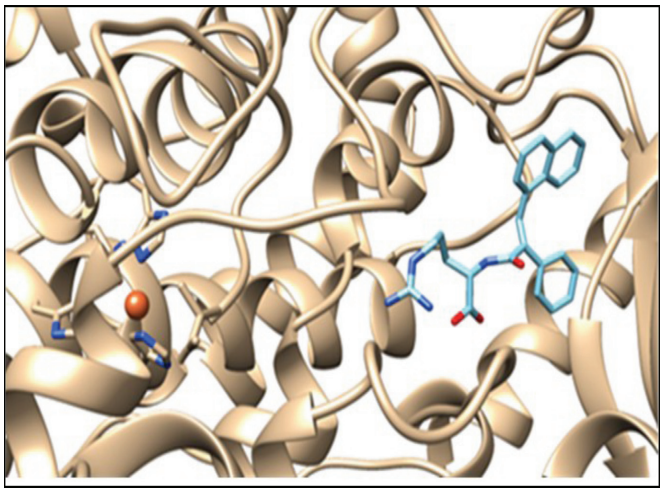


Fig. (1). Docking orientation of 3b (depicted in turquoise) bound to soybean lipoxygenase (LOX-1).

The evaluation of the novel acids against soybean lipoxygenase LOX was accomplished by the UV-based enzyme assay of Pontiki, E. & Hadjipavlou-Litina, D. [17, 18, 44]. This assay may be used as a qualitative or semi-quantitative screening for such activity [45].

The esters **1a-5a**, as well as the nitrooxy ester-ether **5d** and the ether **5c**, did not present any LOX inhibition at 100μM. On the contrary, the amide of naphthyl cinnamic acid **3b** presented a value of IC_{50} = 51μM. In the case of the derivative **4b** in which the phenyl substituent was absent, no inhibition was measured. The biological results were in accordance with the *in silico* study.

Since the inhibition of lipoxygenase occurs *via* a carbon-centered radical on a lipid chain and most of the LOX-inhibitors are antioxidants or free radical scavengers [46], it is possible that **3b** is extended into the hydrophobic domain of LOX, to prevent the access of the substrate to the active site, and hence prevent the lipid peroxidation induced by the enzyme. The result of antioxidant activity for amide **3b** supports the anti-LOX activity.

In silico docking for COX-2 was applied to all the novel derivatives. Compound **5d** was found to be the most potent among the group presenting an AutoDock Vina score (-7.1). It seems that **5d** interacts with the enzyme in an allosteric manner. It is worth to be mentioned that SC-558, a selective COX-2 inhibitor, accommodates in the protein in the same way as **5d** (Fig. **2**) with hydrophobic interactions in an allosteric mode. Considering the *in vitro* assay, only compound **5d** - the nitrooxy ester-ether, presents IC_{50} =82.5μM for COX. This result is according to the modelling findings.

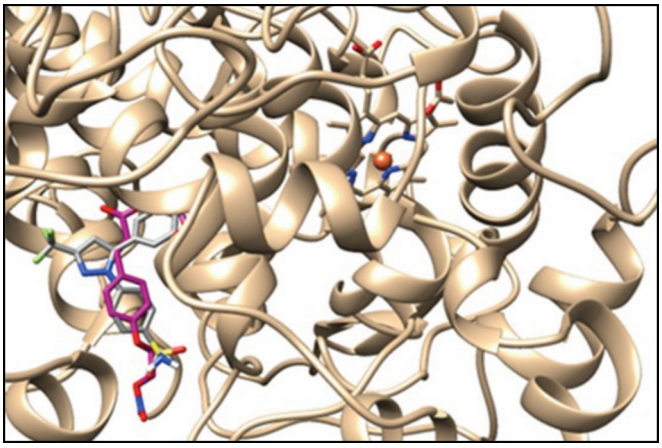


Fig. (2). Docking poses of SC-558 (selective COX-2 inhibitor, depicted in grey) and 5d (depicted in magenta) bound to COX-2.

We decided to study the *in vivo* anti-inflammatory effect of representative compounds using the carrageenin hind paw edema model. Carrageenin has been accepted as a useful phlogistic agent, releases prostaglandins, and is preferred for

seeking new anti-inflammatories, inhibitors of prostaglandin synthesis and COX [28]. Therefore, we considered to study the *in vivo* anti-inflammatory activity of the most potent COX inhibitor, the ether-ester **5d**. Simultaneously, in order to delineate the role of substituent Ar we tested esters **1a**, **4a** and **5a** in order to delineate the role of substituent Ar. Compound **5d** was found to present the highest *in vivo* anti-inflammatory activity within the ester group- followed by 1a, **4a** and **5a**,- which is correlated to its anti-COX activity. The presented activity is higher than the standard drug indomethacin. Lipophilicity does not seem to play any role to this *in vivo* activity. However, the volume of substituent Ar seems to decrease the activity (**4a**, naphthyl). The **3b** was found to be the most potent amide and simultaneously the most active molecule among the tested compounds. The amides 4b and **1b** follow. The naphthyl amides are more potent anti-inflammatories than the phenyl derivatives *e.g.* **4b>1b**. The presence of the naphthyl moiety seems to be crucial for the *in vivo* response. The perusal of the *in vitro*/*in vivo* results points that the **3b** and **5d** can be used as leads for the design of new molecules to target inflammation.

Further experiments are in progress to investigate the role of the L-arginine on the activity.

CONCLUSION

A number of esters, ethers and amides of cinnamic, *p*-coumaric and naphthyl acetic acids, phenyl or not phenyl substituted, a number has been synthesized and subjected to modelling studies. The compounds were studied as inhibitors of COX, LOX, as antioxidants *in vitro* and as anti-inflammatories *in vivo*. The result of antioxidant activity for amide 3b supports the anti-LOX activity. Compounds **3b** and **5d** were found to be potent anti-inflammatories *in vivo* and could be used as leads for the design of new pleiotropic molecules to target inflammation.

LIST OF ABBREVIATIONS

AAPH	=	2,2'-azobis-2-methyl-propanimidamide
Arg	=	Arginine
BOP	=	(Benzotriazol-1-yloxy) *tris* (dimethylmino) Phosphonium Hexafluorophosphate
*c*logP	=	Theoretically Calculated Molar Lipophilicity
COX	=	Cycloxygenase
CPE	=	Carrageenin-induced Paw Edema
DCC	=	*N, N'*-dicyclohexylcarbodiimide
LLA	=	Linoleic Acid
LOX	=	Lipoxygenase
MS	=	Mass Spectrometry
NDGA	=	Nordihydroguaiaretic Acid
NMR	=	Nuclear Magnetic Resonance
NO	=	Nitric Oxide
QSAR	=	Quantitative Structure-activity Relationships
TMPD	=	*N, N, N',N'*-tetramethyl-p-phenylenediamine

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experimental protocols were approved by the Animal Ethics Committee of the Prefecture of Central Macedonia (no. 270079/2500), Greece.

HUMAN AND ANIMAL RIGHTS

No humans are used in this study. Our studies were in accordance with recognised guidelines on animal experimentation. (Guidelines for the care and use of laboratory animals published by the Greek Government 160/1991, based on EU regulations 86/609). Balb/c mice (25-35g, 3-4 months old) were kept in the Centre of the School of Veterinary Medicine (EL54 BIO42), Aristotle University of Thessaloniki, which is registered by the official state veterinary authorities (presidential degree 56/2013, in harmonization with the European Directive 2010/63/EEC).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the manuscript and its supplementary material.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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