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Design and study of some novel ibuprofen derivatives with potential nootropic and neuroprotective properties

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Abstract—Six novel ibuprofen derivatives and related structures, incorporating a proline moiety and designed for neurodegenerative disorders, are studied. They possess anti-inflammatory properties and three of them inhibited lipoxygenase. One compound was found to inhibit cyclooxygenase (COX)-2 production in spleenocytes from arthritic rats. The HS-containing compounds are potent antioxidants and one of them protected against glutathione loss after cerebral ischemia/reperfusion. They demonstrated lipid-lowering ability and seem to acquire low gastrointestinal toxicity. Acetylcholinesterase inhibitory activity, found in two of these compounds, may be an asset to their actions.

1. Introduction

Neurodegenerative disorders of Alzheimer's type (Senile Dementia Alzheimer's Type, SDAT), along with cardiovascular diseases and cancer, are the top causes of morbidity and they are estimated to affect more than 37 million of people worldwide by the year 2020.[1,2] Therefore, it appears interesting to design and synthesise molecules for the treatment of SDAT. Moreover, it is a pharmacochemical challenge, since there is still enough uncertainty about the molecular pathogenesis of nerve cell death[3] and there has not been a really effective intervention yet. However, there is a series of pathobiochemical changes in the demented brain, which could be used as a starting point for rational design of molecules for the particular condition. Thus, the appearance of neurofibrillary tangles intracellularly, amyloid plaques extracellularly, a serious cholinergic deficit, oxidative stress, inflammation, reduction of nerve growth factor, increased apolipoprotein E_4 and hypercholesterolemia are interrelated factors contributing to cell death in SDAT.[3] Consequently, there are several targets for the medicinal treatment of cognition disorders and the potential of multifunctional therapeutic agents for the treatment of complex neurodegenerative diseases has already been considered.[4]

In this paper, we describe the synthesis of derivatives of ibuprofen, a known non-steroidal anti-inflammatory drug which has also been found to affect amyloid pathology in the brain[5] and its long-term use has been associated with reduced risk of neurodegeneration.[6] Ibuprofen is connected, via an amide bond, with a L-proline moiety, expected to contribute to the nootropic properties, since structurally related amides or esters of proline have some nootropic action and proline dipeptides have been found to be about 2000 times more potent than piracetam.[7,8] Proline carboxylic group has further been esterified with 2-methoxy-4-methyl-phenol or 3-(3-hydroxypropyl)pyridine (compounds **1** and **2**, Scheme 1), expected to offer neurotrophic and/or neuroprotective activity, the former by induction of nerve growth factor synthesis,[9] the latter independently of this factor.[10,11] In addition, the carboxylic group of the intermediate proline amide has been converted to amide with cysteamine and L-cysteine ethyl ester (compounds **3** and **4**), known antioxidants with low general toxicity.[12]

Keywords: Ibuprofen derivatives; Inflammation; Antioxidant activity; Cerebral ischemia/reperfusion injury.
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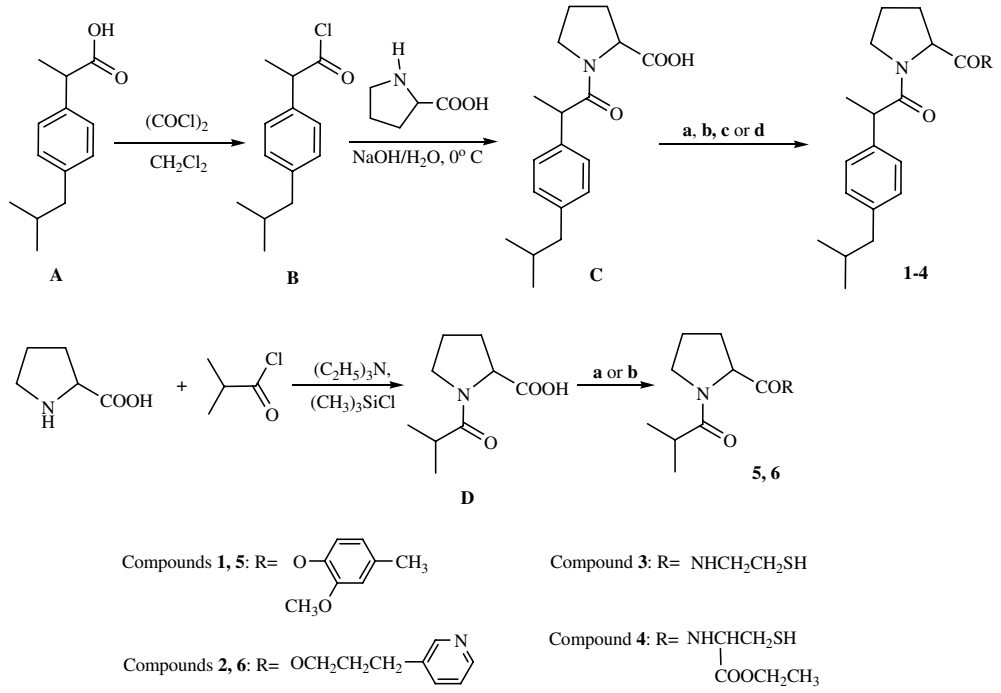

Scheme 1. Synthesis of ibuprofen (A) and related derivatives. Reagents and conditions: (a) 2-methoxy-4-methyl-phenol, DCC/DMAP, room temperature; (b) 3-(3-hydroxypropyl)-pyridine, DCC/DMAP, room temperature; (c) cysteamine, CDI, N_2, 0 °C; (d) L-cysteine ethyl ester, CDI, N_2, 0 °C.

Finally, in order to investigate the importance of the ibuprofen moiety, the L-proline amide with isobutyric acid has been prepared and esterified with 2-methoxy-4-methyl-phenol or 3-(3-hydroxypropyl)pyridine (compounds **5** and **6**).

The anti-inflammatory activity of the synthesised compounds (**1**–**6**) was tested as reduction of paw oedema induced by Freund's complete adjuvant in mice and as in vitro inhibition of lipoxygenase activity. The effect of the administration of a selected compound (**2**) on COX-2 production from spleenocytes from arthritic rats was also studied. Their antioxidant potential was evaluated by the offered inhibition of lipid peroxidation, using rat hepatic microsomal membranes, and by their interaction with 2,2-diphenyl-1-picrylhydrazyl stable radical.

The protective activity of **4**, found to possess significant anti-inflammatory and antioxidant capacity, against oxidative insult to rat brain caused by ischemia–reperfusion, was also tested. Furthermore, the effect of compounds **3** and **4** on cholesterol and triglyceride plasma levels of hyperlipidemic rats was studied. In view of the potential long-term application of these compounds and since ibuprofen, like most non-steroidal anti-inflammatory drugs, has undesired effects mainly from the gastrointestinal tract, the gastrointestinal toxicity of compound **3** was examined. The ability of compounds **1**, **2**, **4** and **6** to inhibit acetylcholinesterase activity in vitro was evaluated.

Finally, the polar surface area of compounds **1**–**6** was calculated as an indicator of their ability to penetrate the blood–brain barrier.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds **1**–**4** is shown in Scheme 1, starting from ibuprofen. As ibuprofen has a chiral centre, two enantiomers exist. Pharmaceutical preparations contain ibuprofen racemate. In the organism, ibuprofen undergoes unidirectional metabolic chiral inversion from (*R*)- to (*S*)-antipode to an extent of 60%.[6]

Ibuprofen [**A**, racemate or the (*S*)-isomer] was converted to the acid chloride (**B**), and then reacted with L-proline [(*S*)-pyrrolidine-2-carboxylic acid], to give the corresponding amide (**C**). Compound **C** was further converted to the final esters (**1** and **2**) or amides (**3** and **4**) by application of conventional methods under mild conditions, with moderate to good yields.

When racemic ibuprofen was used, two diastereomers of compounds **1** and **4** were isolated and only one diastereomer of compounds **2** and **3** was practically obtained. Diastereomers were separated with flash chromatography and identified by comparison with the product yielded from the (*S*)-isomer of ibuprofen. Diastereomer II of **1** [**1**(***SS***)] and diastereomer I of **4** [**4**(***RSR***)] (see Section 4.2) were used in the in vitro and in vivo experiments.

L-Proline reacted with isobutyryl chloride in the presence of chlorotrimethylsilane, to yield the amide **D**, which was esterified to give compounds **5** and **6** (Scheme 1).

2.2. Effect on inflammation induced by Freund's complete adjuvant (FCA)

The effect of compounds **1**–**6** and ibuprofen (racemate) on paw oedema induced by FCA is given in Table 1. FCA paw oedema is a model of acute inflammation in which primarily histamine, serotonin and bradykinin are involved, and secondarily the prostaglandin pathway. The new compounds demonstrated anti-inflammatory activity comparable to that of ibuprofen at the same dose. The most active was compound **2** which, at half the dose of ibuprofen, caused about the same oedema inhibition (58%) with ibuprofen (53%).

Compounds **5** and **6**, which do not contain the complete ibuprofen structure, had no anti-inflammatory activity.

2.3. In vitro effect on lipoxygenase activity

In an attempt to investigate the mechanism of the anti-inflammatory activity of compounds **1**–**6**, their in vitro effect on soybean lipoxygenase was examined. Lipoxygenases are cytosolic nonhaem iron dioxygenases involved in the synthesis of leukotrienes, important inflammatory mediators.[13] Recent findings[14] show that the activation of brain lipoxygenases is an early event in the pathogenesis of Alzheimer's disease. Soybean lipoxygenases convert linoleic acid to 13-hydroperoxylinoleic acid, producing a conjugated diene that absorbs at 234 nm. It is inhibited by non-steroidal anti-inflammatory drugs in a way similar to that of the rat mast cell lipoxygenase and is often used as a reliable tool for testing lipoxygenase inhibitors.[15] Compound **1** demonstrated about 78% inhibition at 0.3 mM, and this effect was time- and concentration-dependent (Fig. 1). The IC_{50} value of compound **1**, after 7 min of incubation, was found to be 0.14 mM, using 250 U/mL of lipoxygenase. Nordihydroguaiaretic acid, a potent lipoxygenase inhibitor, albeit not an anti-inflammatory drug, had an IC_{50} value of 1.3 μM under the same experimental conditions.[16] Thus, lipoxygenase inhibition may, at least partially, contribute to the anti-inflammatory activity of this compound. It is interesting to notice that compound **5**, structurally relevant to **1**, but without the ibuprofen moiety, retained a moderate inhibitory activity (42% at 0.3 mM), indicating that lipoxygenase inhibition by compound **1** is not entirely due to the presence of the ibuprofen structure. At the same concentration, compounds **3**, **4** and **6** had little effect (results not shown). Compound **2** was found practically inactive at 0.2 mM, while at higher concentrations it presented solubility problems during the experiment.

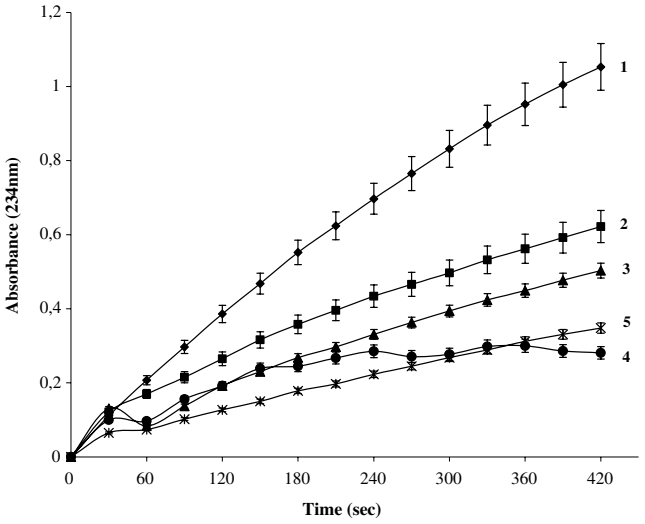

Figure 1. Lipoxygenase activity as affected by compound **1**[*SS*] and (*S*)-ibuprofen. **1**, Control; **2**, 0.1 mM; **3**, 0.2 mM; **4**, 0.3 mM; **5**, ibuprofen 0.3 mM.

Table 1. Effect of ibuprofen (racemate) and the tested compounds on FCA induced inflammation

Compound	Dose (mmol/kg)	Percent oedema increase (means ± SEM)	Percent oedema inhibition
Ibuprofen	0	103.5 ± 25.5	0
	0.30	(44.6 ± 18.4)**	53
1	0	86.2 ± 5.5	0
	0.15	67.9 ± 5.3**	21
	0.30	49.6 ± 8.6*	43
2[*SS*]	0	86.2 ± 5.5	0
	0.15	36.3 ± 5.9**	58
	0.30	16.4 ± 2.0**	81
3[*SS*]	0	70.2 ± 4.1	0
	0.30	27.7 ± 4.9**	61
4	0	102.2 ± 1.5	0
	0.30	68.8 ± 8.1*	33
	0.60	26.5 ± 3.7**	74
5	0	83.5 ± 6.1	0
	0.30	79.3 ± 13.5	1
6	0	83.5 ± 6.1	0
	0.30	77.0 ± 9.7	1

* $P < 0.01$.
** $P < 0.001$ compared to controls (Student's *t*-test).

Since there are not strict structural requirements for lipoxygenase inhibition and a large number of chemicals inhibit lipoxygenase activity, several mechanisms of action have been proposed. However, there is no universally accepted approach to evaluate the relative potency of different substances to cause lipoxygenase inhibition.[17] We could only suggest that the lipophilic character of compounds may be involved in lipoxygenase inhibition, possibly influencing their interaction with the active site.

2.4. Determination of cyclooxygenase (COX)-2 producing spleenocytes

Although a key event in the demented brain is the presence of neuroinflammation, the use of non-steroidal anti-inflammatory drugs for the treatment of Alzheimer's disease patients is complex and still controversial.[18] COX-2 inhibitors have been used in clinical trials for Alzheimer's disease patients, but the results reported so far have been negative.[19] However, there is evidence suggesting a role of COX-2 in synaptic plasticity.[20] Our results (Table 2) confirm the production of COX-2 from spleenocytes of arthritic rats and its inhibition in the animals treated with compound **2**, indicating

Table 2. Effect of compound **2** on COX-2 producing spleenocytes in AID rats

Treatment	Percentage of spleenocytes producing COX-2 (means ± SEM)	
	Day 6	Day 14
Absolute control	0	0
AID	2.50 ± 0.25*	0.25 ± 0.05
AID + compound 2	0.30 ± 0.15*	0.00 ± 0.00

* $P < 0.001$ compared to controls (Student's *t*-test).

that this agent interferes with the production of COX-2 in chronic inflammatory conditions.

2.5. Antioxidant activity

2.5.1. In vitro lipid peroxidation. Oxidative stress, which is induced in Alzheimer's disease, can damage neurons and glia by several mechanisms, increased lipid peroxidation being one of them. Thus, the effect of compounds **1**–**6** on the Fe(II)/ascorbate-induced peroxidation of hepatic microsomal membrane lipids was examined. Compounds **1**, **2**, **5** and **6** had no effect under the conditions of the experiment. On the contrary, compounds **3** and **4** inhibited very strongly the peroxidation reaction, as this is demonstrated in Figure 2A and B. This effect was time- and concentration-dependent. Apparently, this property is due to the presence of the sulfhydryl group in compounds **3** and **4**, which may act via a chain-breaking mechanism.[21] However, applying the above assay, cysteamine and L-cysteine ethyl ester were found to inhibit lipid peroxidation only by 37% and 49%, respectively, at 1 mM,[22] while (*S*)-ibuprofen, L-proline and compounds **C** and **D** were inactive. The most active was **3**, with an IC_{50} value of 19 μM after 45 min of incubation. The IC_{50} of **4** was calculated to be 75 μM under the same conditions. It could be suggested that the difference in activity between **3** and **4** is due to different physicochemical properties. Lipophilicity of compounds is often considered as an important factor contributing to their protection against lipid peroxidation, and this may partially explain the low activity of cysteamine and L-cysteine ethyl ester. However, the most active, compound **3**, is less lipophilic than **4** (*c*log *P* values calculated by the method of Leo–Hansch[23] are 4.07 and 4.50 for **3** and **4**, respectively). Yet, the calculated polar surface area of **3** is smaller than that of **4** (Table 3), possibly enabling its more effective access, retention to and interaction with biological membranes, the site of lipid peroxidation. Furthermore, the availability of the SH group, due to different degrees of conformational freedom, may be an additional important factor for the differences in activity between **3** and **4**, but also between each of them and cysteamine or L-cysteine ethyl ester, respectively.

2.5.2. Interaction with the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The radical scavenging ability of compounds **1**–**6** was also determined as interaction with the hydrophobic, electron accepting radical DPPH.[24] Again, compounds **1**, **2**, **5** and **6** showed no

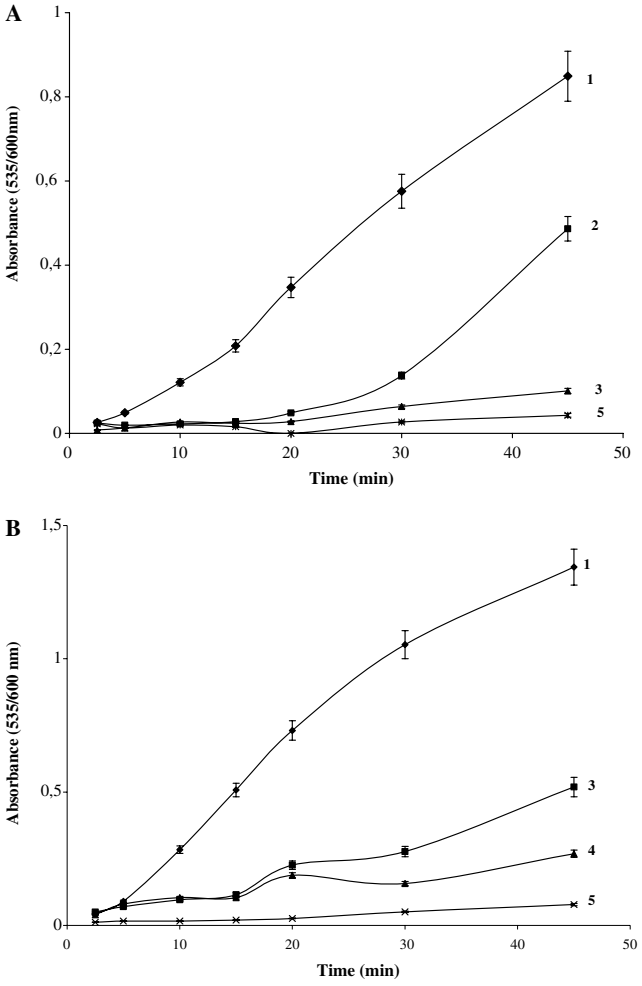

Figure 2. Time course of lipid peroxidation, as affected by various concentrations of compound **3**[***SS***] (A) and compound **4**[***RSR***] (B). **1**, Control; **2**, 0.01 mM; **3**, 0.1 mM; **4**, 0.25 mM; **5**, 0.5 mM.

Table 3. Lipophilicity (*c*log *P*) and polar surface area (TPSA) of compounds

Compound	*c*log *P*	TPSA (Å^2)
1	5.667	55.84
2	5.185	59.50
3	4.066	49.41
4	4.501	75.71
5	2.200	55.84
6	1.710	59.50

interaction, while **3** and **4** interacted very efficiently (Table 4), compound **3** being the most active. The above results are in agreement with these on lipid peroxidation

Table 4. Percent interaction with DPPH (0.2 mM) after 60 min of incubation

Compound (mM)	Percent interaction			
	0.05	0.10	0.20	0.40
3[***SS***]	18	37	76	90
4[***RSR***]	19	38	68	78
Cysteamine	14	37	60	84

and attributed to the presence of the sulfhydryl group. It is known that the interaction of compounds with DPPH is a measure of their reductive ability,[21] which in this case is due to the known reductive properties that a sulfhydryl group conveys.

2.6. Effect on brain oxidative stress caused by ischemia–reperfusion

It has been established that the demented brain suffers from serious oxidative stress,[25] therefore, we found it interesting to study the effect of compound **4** on oxidative stress, challenged by ischemia–reperfusion in rat brain and expressed as malondialdehyde production and glutathione depletion. Ischemia–reperfusion injury is caused by reactive oxygen species produced through Fenton reaction, able to induce lipid peroxidation, as well as by the ensuing inflammation.[26] Compound **4** was selected because it combines significant anti-inflammatory and antioxidant properties with lipophilicity which would enable its access to the CNS, as well as expected lower general toxicity, compared with compounds **1**–**3**, as a derivative of a natural amino acid.

The ischemia–reperfusion injury was well developed under the applied experimental conditions, since lipid peroxidation, expressed as malondialdehyde (MDA) formation, was doubled, while glutathione (GSH) concentration was reduced by about 50%, compared with the control values (Table 5). Treatment with **4** caused a trend to decreased MDA formation towards normal values; however, this effect was not statistically significant. Nevertheless, the effect on glutathione depletion was very significant, since glutathione content was about 90% of the control value. Depletion of total glutathione is a marker for oxidative stress in ischemic brain. Ischemic outcome is aggravated by pharmacological glutathione depletion but improved by administration of glutathione mimetics or precursors.[27] Our results demonstrate that treatment with **4** protects from glutathione depletion caused in the brain by ischemia–reperfusion. This property is probably connected to the antioxidant and reductive ability of **4**, as well as with the anti-inflammatory activity of this compound.[28–30] It has been reported that disturbances of thiol homeostasis are closely related to oxidative stress in the brain[31] and that a decrease of free thiol groups in proteins extracted from hippocampus of patients with Alzheimer's disease is observed, attributed to increased oxidative protein damage, due to insufficiency of the glutathione system in this vulnerable region of the demented brain.[32]

2.7. Effect on plasma cholesterol and triglyceride levels

Increased cholesterol concentration can trigger inflammatory reactions and is a causative factor for age-related disorders such as atherosclerosis and Alzheimer's disease.[33] Hypercholesterolemia is related to neurodegeneration[34] and studies support that amyloid precursor protein processing and brain cholesterol homeostasis are connected,[35] possibly because the low density lipoprotein (LDL) receptor-related protein is localised to membrane regions that also contain secretases involved in the production of Aβ peptide.[36] Additionally, we have reported that several classical non-steroidal anti-inflammatory drugs possess lipid-lowering action.[37] We have also shown that the combination, in a single molecule, of both anti-dyslipidemic and antioxidant properties is an asset for anti-atheromatic action.[38,39] Thus, compounds **3** and **4** as well as the parent drug, ibuprofen, were evaluated for anti-dyslipidemic activity in vivo, and results are shown in Table 6. Both the synthesised compounds decreased total cholesterol, LDL-cholesterol and triglyceride levels more than 50%, compared with the control levels in the plasma of hyperlipidemic rats, compound **4** being more active. Ibuprofen (racemate) decreased these parameters by 32–41%.

It can be summarised that compound **4**, effective against lipid peroxidation and protective against brain oxidative damage, also possessed lipid-lowering ability. Compound **3**, structurally very similar to **4**, also demonstrated almost all the above properties, with small variations. This may

Table 6. Plasma total cholesterol (TC), LDL-cholesterol (LDL-C) and triglyceride (TG) levels after ibuprofen (racemate) and compounds **3**[*SS*] and **4**[*RSR*] treatment

Compound	Plasma values (mg/dl) ± SD (percent reduction compared to hyperlipidemic control group)		
	TC	LDL-C	TG
Hyperlipidemic control group	180 ± 12	56 ± 3	386 ± 30
Ibuprofen	111 ± 8 (38)	38 ± 2 (32)	228 ± 16 (41)
3[***SS***]	54 ± 4 (70)	25 ± 1 (54)	70 ± 5 (82)
4[***RSR***]	29 ± 2 (84)	20 ± 1 (64)	43 ± 3 (89)

In all cases, $P < 0.005$ compared to control group (Student's *t*-test).

Table 5. Protective effect of compound **4**[***RSR***] against oxidative damage during cerebral ischemia/reperfusion

Treatment	MDA nmol/mg protein (means ± SEM)	Percent MDA increase	GSH μmol/g brain (means ± SEM)	Percent GSH decrease
Control	0.80 ± 0.13	0	97.8 ± 6.8	0
Ischemia	1.73 ± 0.31***	116	54.6 ± 8.1***	44
Ischemia + 4[*RSR*]	1.35 ± 0.17**,+	69	90.3 ± 10.0*,++	8

* $P > 0.1$ compared to control group.
** $P < 0.05$.
*** $P < 0.001$.
\+ $P > 0.1$ compared to rats with ischemia (Student's *t*-test).
\++ $P < 0.05$.

indicate an underlying common molecular mechanism, possibly their antioxidant activity.

2.8. Gastrointestinal toxicity

Since ibuprofen, and most of the non-steroidal anti-inflammatory drugs currently in use, have serious gastrointestinal toxicity, intimately connected to their molecular mode of action, we found it necessary to examine one of these compounds, **3**, on a model protocol of this activity which we have reported earlier.[40,41] Furthermore, a prospective nootropic, an agent for long-term application, should be free of this common to the non-steroidal anti-inflammatory drugs' side effect. We selected compound **3**, because it is one of the most active ibuprofen derivatives, which, in addition, contains a cysteamine residue. It has been known for several years that cysteamine itself, at relatively high doses, causes duodenal ulceration,[42] possibly by altering the redox status in duodenal mucosa.[43] Thus, ibuprofen (racemate), cysteamine and compound **3** were given to rats at 1.6 mmol/kg, a dose equal to that at which ibuprofen caused 50% mortality (Table 7). Compound **3** did not cause any mortality or any of the signs of gastrointestinal toxicity observed after ibuprofen or cysteamine administration. This greatly reduced gastrointestinal toxicity may be the result of the entire molecular structure, integrating antioxidant properties, since it is known that oxidative stress is an important component of gastrointestinal ulceration,[44,45] and the elimination of the free carboxylic group of ibuprofen through amide bond formation.

2.9. In vitro effect on acetylcholinesterase activity

The compromised cholinergic system of the demented brain has been well established.[46] Accordingly, the major current approaches to SDAT treatment aim to an increase of acetylcholine in the brain by acetylcholinesterase inhibitors. These agents impede some of the principal processes that contribute to neurodegeneration via acetylcholine-mediated and non-acetylcholine-mediated mechanisms.[47] The inhibition of acetylcholinesterase may be irreversible, mainly via covalent bond formation, or reversible, non-covalent binding. Therefore, we investigated the in vitro effect of compounds **1**, **2**, **5** and **6** on acetylcholinesterase activity, using rat brain homogenates and acetylthiocholine as substrate.[48] Since the applied method is based on the colorimetric determination of thiocholine produced through the catalytic activity of acetylcholinesterase, by its reaction with dithio-bisnitrobenzoic acid, this protocol could not be used with the sulfhydryl-containing compounds **3** and **4**.

Table 7. Gastrointestinal toxicity (1.6 mmol/kg, ip) of ibuprofen (racemate), cysteamine and compound **3** in rats

Compound	Percent mortality	Percent GI ulcers	Body weight change (g/100 g)	Melena incidence
Ibuprofen	50	88	−13	+
Cysteamine	80	40	−7	+
Compound ***3[SS]***	0	0	+3.5	−

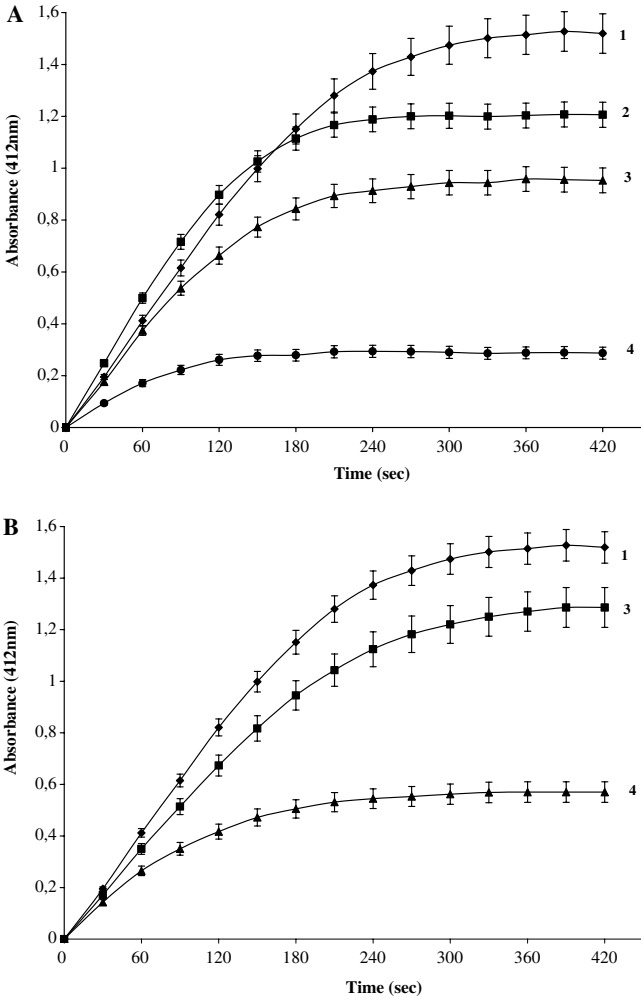

Figure 3. Acetylcholinesterase activity, as affected by (*S*)-ibuprofen, compound **1**[***SS***] (A) and compound **2**[***SS***] (B). **1**, Control; **2**, ibuprofen, 0.6 mM; **3**, 0.3 mM; **4**, 0.6 mM.

The time course of acetylcholinesterase activity, as affected by various concentrations of **1**, **2** and (*S*)-ibuprofen, is shown in Figure 3A and B. At the end of the incubation period, **1**, **2** and ibuprofen offered about 80%, 64% and 20% inhibition, respectively, at 0.6 mM, while **5** and **6** were inactive. Although these are only preliminary results and further investigation is needed, the potential of these compounds to act as acetylcholinesterase inhibitors can be considered beneficial for their prospective nootropic action and may contribute to the mechanisms of action of reported[7,8] structurally related proline derivatives.

2.10. Evaluation of blood–brain barrier (BBB) penetration

The compounds under investigation have been designed to act in the CNS; consequently, a prerequisite for their activity is their ability to pass the blood–brain barrier. A method to predict the ability of compounds for BBB penetration is the calculation of their molecular polar

surface area (PSA), defined as the sum of the surface areas of the polar atoms in a molecule. In a set of 125 different drugs, it has been shown that all those demonstrating CNS activity could be found within a PSA range not higher than 90 $Å^2$.[49] We calculated the topological polar surface area (TPSA) according to the literature[50] and the results are given in Table 3, indicating that all derivatives seem to be capable of entering CNS.

3. Conclusion

With the design of the described ibuprofen derivatives we aimed to compounds that would acquire a series of biological properties able to prevent or restore a number of pathological changes implicated in SDAT and appearing in the demented brain. This study has demonstrated that, in general, the synthesised compounds possess a number of the desired properties. It has been admitted that neurodegenerative disorders, such as Alzheimer's disease, would require multiple drug therapy to address the various pathological disturbances of the disease. Thus, it has been realised that non-steroidal anti-inflammatory drugs, antioxidants, certain cholesterol-lowering agents,[3] drugs that specifically block lipoxygenase activation[14] would be promising agents as parts of a multidrug treatment of Alzheimer's disease. However, the risk of a multidrug combination, with potentially different pharmacokinetic properties of the individual drugs, would be avoided by the development of multifunctional compounds, which, in addition, would offer a simplified treatment to the Alzheimer's disease patients, often showing difficulties in compliance. The synthesised type of compounds may add to this effort.

4. Experimental

4.1. Materials and animals

All commercially available chemicals (from Aldrich-Chemie, Steinheim, Germany; Merck, Darmstadt, Germany) were of the appropriate purity. 2-Thiobarbituric acid, diagnostic kits for total cholesterol, LDL-cholesterol and triglyceride determinations, anti-Rabbit-IgG (A-9169) and anti-Goat-IgG (A-8919) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-COX-2 (sc-1746) was from Santa Cruz Biotechnology (Santa Cruz, USA). Ibuprofen racemate was kindly donated by VIANEX Co. (Greece).

For the in vivo experiments, male Balb-C mice (20–30 g) and Fischer-344 rats (150–220 g) were used.

4.2. Synthesis

Melting points (mp) were obtained on a MEL-TEMP II (Laboratory Devices) apparatus and are uncorrected. Infrared (IR) spectra were taken with a Perkin-Elmer 597 Infrared Spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Brucker 400 MHz spectrometer and chemical shifts are reported in parts per million (δ) relative to tetramethylsilane (TMS). Signals are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet. Elemental analyses were performed with a Perkin-Elmer 2400 CHN analyzer.

4.2.1. 1-(2-(4-Isobutylphenyl)propanoyl)pyrrolidine-2-carboxylic acid, C. A solution of ibuprofen (racemate or the (*S*)-isomer, compound **A**) [48.5 mmol] in dichloromethane [50 mL] was maintained at 0 °C while oxalyl chloride [145.5 mmol] was added dropwise. The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 3 h. The volatile components were removed by distillation under reduced pressure to give 2-(4-isobutylphenyl)propanoyl chloride, **B**, as yellow oil. Compound **B** [46.7 mmol] and a 4 N aqueous solution of sodium hydroxide [12 mL] were then added alternatively, at 0 °C, to an aqueous solution of L-proline [(*S*)-pyrrolidine-2-carboxylic acid], prepared by dissolving L-proline [46.2 mmol] in 2 N aqueous solution of sodium hydroxide [26 mL]. After constant stirring at 0 °C for 20 min and then at room temperature for 1 h, the reaction mixture was washed with ethyl acetate [30 mL], acidified with hydrochloric acid 1 N to pH 2, extracted with chloroform [4× 60 mL] and dried [Na_2SO_4]. The solvent was evaporated under reduced pressure and the residue was recrystallised from ether and petroleum ether, giving a white solid (compound **C**), which was further used without separation of diastereomers at this stage, due to their similar chromatographic properties. Yield 90%, mp 89–93 °C.

Starting from (*S*)-ibuprofen and L-proline, (*S*)-1((*S*)-2-(4-isobutylphenyl)propanoyl)pyrrolidine-2-carboxylic acid was obtained, Mp 112–114 °C. IR, 1752, 1598 cm^{-1}. ^{1}H NMR: ($CDCl_3$), δ 0.83 (d, 6H, $CH(CH_3)_2$), δ 1.39 (d, 3H, CH_3CHCO), δ 1.76–1.94 (m, 4H, C-3 pyrr, C-4 pyrr), δ 2.27–2.39 (m, 3H, $CH(CH_3)_2$, $CH_2CH(CH_3)_2$), δ 3.17–3.46 (m, 2H, C-5 pyrr), δ 3.69 (q, 1H, CH_3CHCO), δ 4.58 (t, 1H, C-2 pyrr) δ 7.01–7.10 (dd, 4Harom), δ 10.32 (s, 1H, OH). Analysis for $C_{18}H_{25}NO_3$ calculated: C 71.24%, H 8.31%, N 4.62%. Found: C 71.04%, H 8.53%, N 4.72%.

4.2.2. (*R*)-1-(Isobutyryl)pyrrolidine-2-carboxylic acid, D. To a suspension of L-proline [40 mmol] in dichloromethane [280 mL], triethylamine [108 mmol] and chlorotrimethylsilane [48 mmol] were added successively and the mixture was stirred for 3 h at room temperature. Then, isobutyryl chloride [20 mmol] was added at 0 °C and the mixture was stirred for 48 h at room temperature, concentrated under reduced pressure, the residue was dissolved in aqueous K_2CO_3 solution [5%, 50 mL], washed with diethyl ether [60 mL], cooled, acidified to pH 2 with 6 M hydrochloric acid, extracted with ethyl acetate [3× 40 mL] and dried [Na_2SO_4]. The solvent was evaporated under reduced pressure and the residue was recrystallised from ethyl acetate and petroleum ether. Yield 46%, mp 118–120 °C. IR, 3300–2500, 1718, 1598 cm^{-1}. ^{1}H NMR: ($CDCl_3$), δ 1.07 (d, 6H, $CH(CH_3)_2$), δ 1.90–2.29 (m, 4H, C-3pyrr, C-4pyrr), δ 2.65 (m, 1H, $CH(CH_3)_2$), δ 3.46–3.65 (m, 2H, C-5pyrr), δ 4.50 (t, 1H, C-2pyrr), δ 10.76 (s, 1H, OH). Analysis for

$C_9H_{15}NO_3$ calculated: C 58.36%, H 8.16%, N 7.56%. Found: C 58.59%, H 8.03%, N 7.83%.

4.2.3. Synthesis of esters and amides of compounds C and D. Compounds **1**–**6** were synthesised by esterification or amidation of **C** or esterification of **D** with the proper alcohol or amine. Because of the use of racemic ibuprofen, L-proline [(*S*)-pyrrolidine-2-carboxylic acid], and L-cysteine ethyl ester [(*R*)-ethyl 2-amino-3-mercaptopropanoate, for compound **4**], two diastereomers of **1** and **4** were synthesised, which were separated and identified. The applied synthetic method and purification procedure for compounds **2** and **3**, however, resulted in practically only one product, identified to be the (*S*)-3-(pyridin-3-yl)propyl 1-((*S*)-2-(4-isobutylphenyl)-propanoyl)pyrrolidine-2-carboxylate diastereomer of **2** and the (*S*)-1-(*S*)-2-(4-isobutylphenyl)propanoyl-*N*-(2-mercaptoethyl)pyrrolidine-2-carboxamide diastereomer of **3**.

4.2.4. General procedure for the esters 1, 2, 5 and 6. A solution of compound **C** [10 mmol], 2-methoxy-4-methyl-phenol [11 mmol] (**1**) or 3-(3-hydroxypropyl)-pyridine [11 mmol] (**2**), *N*,*N*′-dicyclohexylcarbodiimide [DCC, 11 mmol] and *N*,*N*-dimethylaminopyridine [DMAP, 1 mmol] in dichloromethane was maintained at room temperature for 3 h. After filtration, the filtrate was washed with water, then with a 5% aqueous solution of acetic acid, again with water and dried [$MgSO_4$]. The solvent was evaporated under reduced pressure and the residue was dissolved in dry ethyl acetate, left at 4 °C for 24 h, filtered and evaporated under reduced pressure.

The esters **5** and **6** of compound **D** were prepared from **D** and 2-methoxy-4-methyl-phenol (**5**) or 3-(3-hydroxypropyl)-pyridine (**6**) according to the method described for **1** and **2**.

4.2.5. 2-Methoxy-4-methylphenyl 1-(2-(4-isobutylphenyl)propanoyl)pyrrolidine-2-carboxylate, 1. The use of ibuprofen racemate and L-proline resulted in two diastereomers of compound **1**, well separated in TLC. They were isolated by flash chromatography (petroleum ether/ethyl acetate 4:1) in a molecular ratio almost 1:1. The less polar (diastereomer I) is a white solid with mp 78–82 °C. The other diastereomer (diastereomer II) is a liquid. After the synthesis of compound **1**, beginning with (*S*)-ibuprofen, it was found that diastereomer I is (*S*)-2-methoxy-4-methylphenyl 1((*R*)-2-(4-isobutylphenyl)propanoyl)pyrrrolidine-2-carboxylate [**1**(***SR***)], and diastereomer II is (*S*)-2-methoxy-4-methylphenyl 1((*S*)-2-(4-isobutylphenyl)propanoyl)pyrrrolidine-2-carboxylate [**1**(***SS***)]. Yield for both diastereomers 37%. IR, 1769, 1659 cm^{-1}. ^1H NMR: Diastereomer I [**1**(***SR***)] ($CDCl_3$), δ 0.87 (d, 6H, $CH(CH_3)_2$), δ 1.42 (d, 3H, CH_3CHCO), δ 1.82 (m, 2H, C-4 pyrr), δ 2.13 (m, 3H, C-3 pyrr, $CH(CH_3)_2$), δ 2.31 (s, 3H, phenyl-CH_3), δ 2.42 (d, 2H, $CH_2CH(CH_3)_2$), δ 3.27 (m, 2H, C-5 pyrr), δ 3.71 (m, 1H, CH_3CHCO), δ 3.78 (s, 3H, OCH_3), δ 4.68 (m, 1H, C-2 pyrr), δ 6.99–7.24 (m, 7Harom). Diastereomer II [**1**(***SS***)] ($CDCl_3$): δ 0.82 (d, 6H, $CH(CH_3)_2$), δ 1.39 (d, 3H, CH_3CHCO), δ 1.72–2.40 (m, 10H, C-3 pyrr, C-4 pyrr, $CH(CH_3)_2$, phenyl-CH_3, $CH_2CH(CH_3)_2$), δ 3.45 (m, 2H, C-5 pyrr), δ 3.69–3.78 (m, 4H, CH_3CHCO, OCH_3), δ 4.72 (m, 1H, C-2 pyrr), δ 6.67–6.75 (m, 2Harom), δ 6.85–7.17 (m, 5Harom). Analysis for $C_{26}H_{33}NO_4$: diastereomer I [**1**(***SR***)], calculated: C 73.73%, H 7.86%, N 3.31%. Found: C 73.59%, H 8.22%, N 3.84%. Diastereomer II [**1**(***SS***)], calculated: C 73.73%, H 7.86%, N 3.31%. Found: C 73.43%, H 8.22%, N 3.41%.

4.2.6. 3-(Pyridin-3-yl)propyl 1-(2-(4-isobutylphenyl)propanoyl)pyrrolidine-2-carboxylate, 2. It was isolated as practically the only diastereomer, by flash chromatography (petroleum ether/ethyl acetate 1:2), as a liquid. It was identified as described for **1**, and found to be (*S*)-3-(pyridin-3-yl)propyl 1-(*S*)-(2-(4-isobutylphenyl)propanoyl)pyrrolidine-2-carboxylate [**2**(***SS***)]. Yield 39%. IR, 1739, 1645 cm^{-1}. ^1H NMR: ($CDCl_3$), δ 0.87 (d, 6H, $CH(CH_3)_2$), δ 1.39 (d, 3H, CH_3CHCO), δ 1.80–2.01 (m, 7H, $CH_2CH_2CH_2$, $CH(CH_3)_2$, C-3 pyrr, C-4 pyrr), δ 2.37 (d, 2H, $CH_2CH(CH_3)_2$), δ 2.64 (m, 2H, CH_2-pyridyl), δ 3.23 (m, 2H, C-5 pyrr), δ 3.64–3.75 (m, 1H, CH_3CHCO), δ 4.08–4.17 (m, 2H, OCH_2), δ 4.41 (t, 1H, C-2 pyrr), δ 7.05 (d, 1Harom), δ 7.15 (m, 3H, 2Harom, C-5 pyridyl), δ 7.49 (d, 1H, C-4 pyridyl), δ 8.42 (m, 2H, C-2 pyridyl, C-6 pyridyl). Analysis for $C_{26}H_{34}N_2O_3$ calculated: C 73.89%, H 8.11%, N 6.63%. Found: C 73.73%, H 8.37%, N 6.92%.

4.2.7. 1-(2-(4-Isobutylphenyl)propanoyl)-*N*-(2-mercaptoethyl)pyrrolidine-2-carboxamide, 3. A solution of compound **C** [13.2 mmol] in *N*,*N*-dimethylformamide [DMF, 4 mL] dried over molecular sieve 4 Å was maintained at 0 °C under nitrogen, while 1,1′-carbonyldiimidazole [CDI, 13.7 mmol] was added. When the vigorous release of carbon dioxide stopped, the mixture was further stirred at room temperature until carbon dioxide was no longer released. Subsequently, cysteamine (2-aminoethanethiol) hydrochloride [13.3 mmol] was added and the reaction mixture was stirred for 1 h. Then, water [20 mL] was added; the mixture was maintained at room temperature under nitrogen for 48 h, extracted with chloroform [3× 30 mL] and dried [Na_2SO_4]. The solvent was evaporated under reduced pressure and, with this procedure, compound **3** was finally isolated as the only diastereomer by flash chromatography (petroleum ether/ethyl acetate 1:1) as a white solid. It was identified as described for **1**, and found to be (*S*)-1-(*S*)-(2-(4-isobutylphenyl)propanoyl)pyrrrolidine-*N*-(2-mercaptoethyl)pyrrolidine-2-carboxamide [**3**(***SS***)]. Yield 40%, mp 75–78 °C. IR, 1628, 2363, 3282, 3432 cm^{-1}. ^1H NMR: ($CDCl_3$), δ 0.86 (d, 6H, $CH(CH_3)_2$), δ 1.31 (t, 1H, SH), δ 1.44 (d, 3H, CH_3CHCO), δ 1.78–2.04 (m, 5H, C-3 pyrr, C-4 pyrr, $(CH_3)_2CH$), δ 2.33–2.64 (m, 4H, CH_2SH, $(CH_3)_2CHCH_2$), δ 3.23–3.75 (m, 5H, C-5 pyrr, $NHCH_2$, CH_3CHCO), δ 4.51–4.66 (m, 1H, C-2 pyrr), δ 7.05–7.24 (m, 4Harom), δ 7.47 (s, 1H, NH). Analysis for $C_{20}H_{30}N_2O_2S$ calculated: C 66.26%, H 8.35%, N 7.78%. Found: C 66.69%, H 8.54%, N 7.46%.

4.2.8. Ethyl 2-(1-(2-(4-isobutylphenyl)propanoyl)pyrrolidine-2-carboxamido)-3-mercaptopropanoate, 4. A solution of compound **C** [6.6 mmol] in dry *N*,*N*-dimethylformamide [4 mL] was maintained at 0 °C under

nitrogen, while CDI [6.8 mmol] was added. The mixture was stirred at room temperature until carbon dioxide was no longer released. Then, L-cysteine ethyl ester [(*R*)-ethyl 2-amino-3-mercaptopropanoate] hydrochloride [6.7 mmol] was added and the reaction mixture was maintained at room temperature for 24 h. The mixture was washed with water twice, chloroform was added and the organic phase was dried [$CaCl_2$]. The solvent was evaporated under reduced pressure and the residue was flash chromatographed (petroleum ether/ethyl acetate 3:1) for the separation of isomers. Both diastereomers are colourless oils. After the synthesis of compound **4**, beginning with (*S*)-ibuprofen, it was found that the less polar diastereomer I is (*R*)-ethyl 2-((*S*)-1-((*R*)-2-(4-isobutylphenyl)propanoyl)pyrrolidine-2-carboxamido)3-mercaptopropanoate [**4**(***RSR***)] and diastereomer II is (*R*)-ethyl 2-((*S*)-1-((*S*))-2-(4-isobutylphenyl)- propanoyl)pyrrrolidine-2-carboxamido-3-mercaptopropanoate [**4**(***RSS***)]. Yield 32% for diastereomer I, and very poor (less than 10%) for diastereomer II. IR, 1632, 1683, 1740, 2560 cm^{-1}. ^1H NMR: Diastereomer I [**4**(***RSR***)] ($CDCl_3$), δ 0.87 (d, 6H, $CH(CH_3)_2$), δ 1.28 (t, 3H, OCH_2CH_3), δ 1.43 (d, 3H, CH_3CHCO), δ 1.67–2.26 (m, 6H, **SH**, C-3 pyrr, C-4 pyrr, $(CH_3)_2CH$), δ 2.41 (d, 2H, $(CH_3)_2CHCH_2$), δ 2.90–3.08 (m, 2H, C-5 pyrr), δ 3.19–3.27 (m, 2H, CH_2SH), δ 3.76 (q, 1H, CH_3CHCO), δ 4.21 (m, 2H, OCH_2), δ 4.54 (m, 1H, C-2 pyrr), δ 4.75–4.79 (m, 1H, NHCH), δ 7.06 (d, 2Harom), δ 7.16 (d, 2Harom), δ 7.54 (d, 1H, NH). Analysis for $C_{23}H_{34}N_2O_4S$ $1.1H_2O$ calculated: C 60.79%, H 8.03%, N 6.16%. Found: C 60.41%, H 7.98%, N 6.39%.

4.2.9. (*R*)-2-Methoxy-4-methylphenyl 1-(isobutyryl)pyrrolidine-2-carboxylate, 5. It was isolated by flash chromatography (petroleum ether/ethyl acetate 2:1) as a yellow oil. Yield 60%. IR, 1769, 1650, 1508 cm^{-1}. ^1H NMR: ($CDCl_3$), δ 1.07 (d, 6H, $CH(CH_3)_2$), δ 1.96–2.37 (m, 7H, C-3pyrr, C-4pyrr, phenyl-CH_3), δ 2.56–2.67 (m, 1H, $CH(CH_3)_2$), δ 3.51–3.73 (m, 5H, C-5pyrr, OCH_3), δ 4.65 (t, 1H, C-2pyrr), δ 6.65–6.92 (m, 3Harom). Analysis for $C_{17}H_{23}NO_4$ calculated: C 66.86%, H 7.59%, N 4.59%. Found: C 66.80%, H 7.65%, N 4.49%.

4.2.10. (*R*)-3-(Pyridin-3-yl)propyl 1-(isobutyryl)pyrrolidine-2-carboxylate,6. It was isolated by flash chromatography (ethyl acetate) as yellow oil. Yield 32%. IR, 1743, 1645, 1577 cm^{-1}. ^1H NMR: ($CDCl_3$), δ 1.04 (d, 6H, $CH(CH_3)_2$), δ 1.86–2.33 (m, 6H, C-3pyrr, C-4pyrr, CH_2-CH_2CH_2), δ 2.58–2.65 (m, 3H, $CH(CH_3)_2$, pyridyl-CH_2), δ 3.47–3.64 (m, 2H, C-5pyrr), δ 4.04 (m, 2H, OCH_2), δ 4.40 (t, 1H, C-2pyrr), δ 7.13 (d, 1H, C-4pyridyl), δ 7.45 (m, 1H, C-5pyridyl), δ 8.37 (d, 2H, C-2pyridyl, C-6pyridyl). Analysis for $C_{17}H_{24}N_2O_3$ 0.4 H_2O calculated: C 65.53%, H 8.02%, N 8.99%. Found: C 65.34%, H 8.27%, N 9.00%.

4.3. Effect on inflammation induced by Freund's complete adjuvant (FCA)

Oedema was induced by the intradermal (id) injection of FCA [0.05 mL/paw] into the right hind paw of mice, the left paw serving as control. The test compounds, suspended in water with few drops of Tween 80, were given intraperitoneally (ip) [0.15–0.60 mmol/kg body weight] 15 min before the FCA injection. Three hours later, the hind paws were excised and weighted separately. The produced oedema was estimated as paw weight increase compared to the control animals that received only the liquid vehicle.[51]

4.4. In vitro effect on lipoxygenase activity

The reaction mixture [total volume 3 mL] contained 100 μL of the test compounds [0.1–0.3 mM], dissolved in 60% aqueous ethanol (sample), or 100 μL of the solvent (reference) and 200 μL of soybean lipoxygenase [250 U/mL] in 2.6 mL of Tris buffer (pH 9). The reaction was initiated by the addition of 100 μL sodium linoleate [0.1 mM] in the sample mixture, an equal volume of buffer being added to the reference solution, and monitored for 7 min at 28 °C, by recording the absorbance of a conjugated diene structure at 234 nm, due to the formation of 13-hydroperoxylinoleic acid.[52]

4.5. Effect on COX-2 production in rat spleenocytes

Adjuvant arthritis (AID) was induced in rats by id injection of FCA [0.1 mL] to the third distal of the tail, as previously described.[53] The following day and for 10 days, compound **2**, suspended in water with few drops of Tween 80, was given ip [0.15 mmol/kg body weight]. Animals were sacrificed on the sixth and the 14th day post AID treatment, spleens were removed quickly, immersion-fixed at 4 °C for 24 h, dehydrated, embedded in paraffin and cut into about 15 (5 μm thick) sections. Tissues were then deparaffinised with toluene, rehydrated, treated with H_2O_2 [0.1% in methanol] for 30 min, incubated in 5% foetal bovine serum (FBS) in phosphate buffer saline (PBS) for 1 h, then incubated with the primary antibody for 1 h at a concentration of 10 μg/mL. After washing, slices were incubated for 1 h with the appropriate peroxidase-conjugated secondary antibody (diluted 500 times in PBS). Finally, slices were incubated for 10 min in a dianisidine solution [5 mg of dianisidine, 1 mL methanol, 9 mL PBS and 5 μL H_2O_2 0.1%]. Red–orange areas, representing positive areas, were visualised using the appropriate microscopic equipment.[54]

4.6. In vitro lipid peroxidation

Hepatic microsomal fraction from untreated rats was prepared.[38] The incubation mixture contained heat-inactivated (90 °C for 90 s) microsomal fraction, corresponding to 2.5 mg protein/mL (final concentration) or 4 mM fatty acid residues,[55] ascorbic acid [0.2 mM] in Tris–HCl/KCl buffer [50/150 mM] and the test compounds dissolved in dimethylsulfoxide. The peroxidation reaction was initiated by the addition of a freshly prepared $FeSO_4$ solution [10 μM] and the mixture was incubated at 37 °C. Aliquots [0.3 mL] were taken at various time intervals for 45 min. Lipid peroxidation was assessed spectrophotometrically (535 against 600 nm) by the determination of 2-thiobarbituric acid reactive material.[39] All compounds as well as dimethylsulfoxide were tested and found not to interfere with the assay.

4.7. Interaction with the stable radical DPPH

The test compounds were dissolved in absolute ethanol (analytical grade, iron content less than 10^{-5}% w/v), added to an equal volume of an ethanolic solution of DPPH [final concentration 0.2 mM] at various concentrations [25–400 μM] and incubated at 22 ± 2 °C. Absorbance (517 nm) was recorded after 30 min.[29]

4.8. Effect on brain oxidative stress caused by ischemia–reperfusion

Compound **4** [0.3 mmol/kg body weight] was administered ip once per day for 4 days and once more 2 h before the initiation of ischemia. The common carotid arteries were exposed and clamped with aneurysm clips to induce incomplete cerebral ischemia. Animals were subjected to 45 min of carotid occlusion, then the clips were removed and brain was reperfused for 90 min. Animals were subsequently sacrificed, brains were removed quickly, homogenised [1 g in 10 mL isotonic KCl] for 1 min in an ice-cold solution of KCl [1.19% w/v] and stored at −80 °C for further evaluation.

4.8.1. Brain lipid peroxidation. Lipid peroxidation of rat brain homogenates was determined fluorometrically as 2-thiobarbituric acid reactive substance.[28] The degree of lipid peroxidation was expressed as malondialdehyde formation (nmol/mg brain protein), using malondialdehyde bis(dimethyl acetal) as reference.

4.8.2. Brain glutathione levels. The determination of glutathione was performed in the brain homogenate supernatant (9000*g*, 4 °C, 20 min). Reduced glutathione was determined based on its reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), in the presence of NADPH and glutathione reductase. Absorbance (412 nm) was recorded after 3 min of incubation.[56]

4.9. Effect on plasma cholesterol and triglyceride levels

An aqueous solution of Triton WR 1339 was given ip to rats [200 mg/kg], and the examined compounds [0.3 mmol/kg] were administered ip 30 min later. After 24 h, blood was taken from the aorta and used for the determination of plasma cholesterol, LDL-cholesterol and triglyceride concentration.[39]

4.10. Gastrointestinal toxicity

Compound **3**, cysteamine hydrochloride and ibuprofen (racemate) [1.6 mmol/kg] were administered subcutaneously (sc) to rats once daily for 4 days. Perforating gastrointestinal ulcers, melena defecation, body weight change and mortality were recorded 24 h after the last injection.[40]

4.11. Determination of acetylcholinesterase activity

Brains from untreated rats were homogenised [20 mg/mL] in phosphate buffer [0.1 M, pH 8]. Acetylcholinesterase activity was assessed using brain homogenate, acetylthiocholine [0.5 mM] as a substrate, in the presence of the tested compounds (dissolved in 60% ethanol) and evaluating the reaction product of the liberated thiocholine with DTNB at 412 nm.[48] The used solvent system was tested and found not to interfere with the assay. Physostigmine sulfate, used as a standard, caused a 56% inhibition of acetylcholinesterase at 0.15 μM, under the same experimental conditions.

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