Full Paper

Ibuprofen and Lipoic Acid Diamides as Potential Codrugs with Neuroprotective Activity

Piera Sozio[1], Eleonora D'Aurizio[1], Antonio Iannitelli[1], Amelia Cataldi[2], Susi Zara[2], Franco Cantalamessa[3], Cinzia Nasuti[3], and Antonio Di Stefano[1]

[1] Dipartimento di Scienze del Farmaco, Università "G. D'Annunzio", Chieti, Italy
[2] Dipartimento di Biomorfologia, Università "G. D'Annunzio", Chieti, Italy
[3] Dipartimento di Medicina Sperimentale e Sanità Pubblica, Università di Camerino, Camerino (MC), Italy

Current evidences support the hypothesis that non-steroidal anti-inflammatory drugs (NSAIDs) and antioxidant therapy might protect against the development of Alzheimer's disease (AD). In the present work, our attention was focused on ibuprofen (IBU) used in clinical trails to prevent Alzheimer's disease, and (*R*)-α-lipoic acid (LA) considered as a potential neuroprotective agent in AD therapy. In particular, we investigated a series of lipophilic molecular combinations obtained by joining (*R*)-α-lipoic acid and ibuprofen via an amide bond. These new entities might allow targeted delivery of the parent drugs to neurons, where cellular oxidative stress and inflammation seem related to Alzheimer's disease. Our study included the synthesis of conjugates **1**–**3** and the evaluation of their physicochemical and *in-vitro* antioxidant properties. The new compounds are extremely stable in aqueous buffer solutions (pH = 1.3 and 7.4), and in rat and human plasma they showed a slow bioconversion to ibuprofen and (*R*)-α-lipoic acid. Codrugs **1**–**3** displayed *in vitro* free radical scavenging activity and were hydrolyzed more rapidly in brain tissue than in rat serum indicating that these new entities might allow targeted delivery of the parent drugs to neurons. The immunohistochemical analysis of Aβ (1-40) protein showed that Aβ-injected cerebral cortices treated with ibuprofen or compound **1** showed few plaques within capillary vessels and, in particular, Aβ (1-40) protein was less expressed in codrug-**1**-treated than in ibuprofen-treated cerebral cortex.

Keywords: Alzheimer's disease / Codrug / (R)-α-Lipoic acid / Neuroprotective agent

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Introduction

Alzheimer's disease (AD) is an irreversible progressive neurodegenerative disorder of the central nervous system (CNS) that gradually destroys patient memory and cognition in the geriatric population [1]. Much evidence gathered over the last 30 years has indicated that plaques in the AD brain are associated with numerous markers for inflammation, including activated astrocytes, reactive microglia, and interleukins [2]. The senile plaque, the hallmark of AD, is characterized by extracellular deposition of amyloid β peptide (Aβ) in hippocampal and cerebral cortical regions accompanied by the presence of intracellular neurofibrillary tangles that occupy much of the cytoplasm of pyramidal neurons; in addition, microglia and astroglia are present near the plaque, where numerous inflammation factors are overexpressed. It is thought that the inflammatory process, including superoxide production ("oxidative burst"), is an important source of oxidative stress in AD patients. [3] This hypothesis suggests that reactive oxygen species (ROS) and reac-

Correspondence: Antonio Di Stefano, Dipartimento di Scienze del Farmaco, Università "G. D'Annunzio", Via dei Vestini 31, 66100 Chieti, Italy.
E-mail: adistefano@unich.it
Fax: +39 871 355-4706

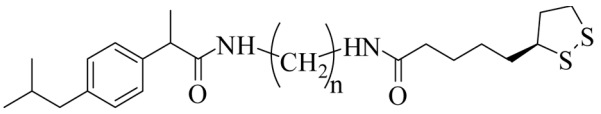
Abbreviations: Alzheimer's disease (AD); amperometric electrode array detector (AEAD); amyloid β peptide (Aβ); blood-brain barrier (BBB); diode-array detection (DAD); 1,1-diphenyl-2-picrylhydrazyl (DPPH); deoxyribose oxidation method (DOM); 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDCI); hydroxybenzotriazole (HOBT); ibuprofen (IBU); dihydrolipoic acid (DHLA); (*R*)-α-lipoic acid (LA); malondialdehyde (MDA); reactive nitrogen species (RNS); reactive oxygen species (ROS); thiobarbituric acid (TBA)

tive nitrogen species (RNS) generated intracellularly and extracellularly by various mechanisms, are the major risk factors that initiate and promote neurodegeneration in idiopathic AD; DNA damage induced by free radicals or enzymatic activity modifications can trigger the initiation of cell death. These observations suggest that the oxidative damage leading to accumulation of DNA errors may be an important factor in the progression of neuronal loss in AD [4].

Many evidences suggest that non-steroidal anti-inflammatory drugs (NSAIDs) might protect against the development of AD, substantially delaying its onset, given the importance of the effects of inflammatory processes in the brain of AD patients. [5] Furthermore, Weggen *et al*. investigating the effect of various NSAIDs on the production of Aβ (1–42) in cell culture reported that not all NSAIDs affected its production, noting that it seemed not to be mediated by inhibition of cyclooxygenase (COX) activity, the principal pharmacological target of NSAIDs [6]. In particular, these investigations found that ibuprofen (IBU), indomethacin, and sulindac sulphide reduced Aβ (1–42) production, while naproxen, aspirin, meloxicam, and celecoxib did not have this effect. The proposed mechanism for this activity is an allosteric modulation of γ-secretase activity, the enzyme responsible for the formation of Aβ. Crucial factors in the efficiency of these drugs as neuroprotective agents seem to be their high plasma-binding percentages and apparently low distribution volumes, as well as the blood-brain barrier (BBB) [7, 8].

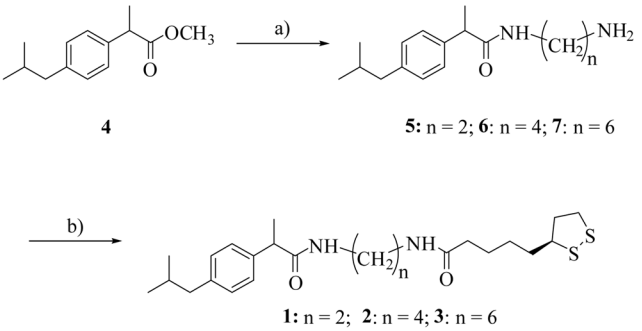
Antioxidant therapy has shown a slight ameliorating effect on the progression of AD; in particular, epidemiological studies have shown that use of vitamins E and C decreases the risk of AD [9]. However, the use of these natural antioxidants as therapeutic agents is limited, mainly due to the marginal efficiency of these scavengers in crossing the BBB.

(*R*)-α-Lipoic acid (LA) has been used in clinical trails for the treatment of diabetic neuropathy and has been used in trials to prevent AD [10]. LA is a dithiol compound normally bound to lysine residues of mitochondrial α-keto acid dehydrogenases that readily crosses the BBB and accumulates in all neuronal cell types [11]. There, cytosolic and mitochondrial dehydrogenases rapidly reduce it to dihydrolipoic acid (DHLA), the active compound responsible for most of the beneficial effects against AD, such as the increase of acetylcholine (ACh) production by activation of choline acetyltransferase, the chelation of redox-active transition metals, the increase of reduced glutathione levels, and the downregulation of redox-sensitive inflammatory processes [12].



1: n = 2 **2**: n = 4 **3**: n = 6

Figure 1. Chemical structures of multifunctional codrugs **1–3**.



4 **5:** n = 2; **6**: n = 4; **7**: n = 6

1: n = 2; **2**: n = 4; **3**: n = 6

Reagents: **a**) alkyldiamine; **b**) CH_2Cl_2, LA, TEA, HOBT, EDCI.

Scheme 1. Synthesis of compounds **1–6**.

Starting from these data and in order to enhance the brain availability of NSAIDs, we investigated lipophilic molecular combinations obtained by joining an antioxidant molecule with a NSAID. In this work, our attention was focused on IBU, considered as a potential neuroprotective agent in AD therapy able to reduce Aβ brain levels through the inhibition of pro-amyloidogenic factors [13, 14]. IBU was linked to LA, whose benefits in AD treatment have been previously demonstrated [15, 16]; these compounds might permit targeted delivery of NSAID and LA directly to neurons, where cellular stress and inflammation are associated with AD. In order to modify the physicochemical properties of IBU for improved BBB crossing, and, in addition, to take advantage of the apparent synergic mechanism of IBU and LA, here, we propose the synthesis of molecular combinations (codrugs **1–3**) in which IBU is covalently linked via an amide bond to LA (Fig. 1). The new codrugs **1–3** (for their synthesis, see Scheme 1), with a high degree of chemical and enzymatic stability under physiological conditions, can afford more efficacious CNS delivery than can LA and IBU alone. Specifically, this study included the synthesis of codrugs **1–3** and the evaluation of their physicochemical and biological properties. The pharmacological effects of selected compounds **1** in a Aβ-infused AD rat model were also investigated.

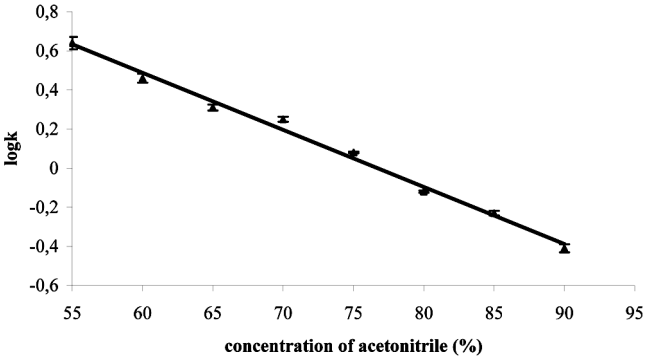


Figure 2. Dependence between logk values and concentration of acetonitrile in mobile phase for compound **1**.

Table 1. Physicochemical properties of codrugs **1–3**.

Compound	cLogP[§]	Logk$_0$[§]	Solubility in water[§](mg/mL)
1	4.43 (± 0.53)	2.239 (± 0.090)	0.02 (± 0.9 × 10^{-3})
2	4.95 (± 0.51)	2.387 (± 0.095)	0.02 (± 0.2 × 10^{-3})
3	5.71 (± 0.50)	2.719 (± 0.057)	0.01 (± 0.4 × 10^{-3})
IBU	3.72 (± 0.23)	1.319 (± 0.047)	–
LA	2.16 (± 0.29)	1.120 (± 0.009)	–

§ Values are means of three experiments, standard deviation (S.D.) is given in parentheses.

Results and discussion

Lipophilicity is an essential feature for predicting the penetration of a molecule through the BBB; this property was estimated using reverse-phase chromatographic retention times (RT) [17]; log capacity factor (logK_0) values were determined using a mixture of acetonitrile and water as eluant. The lipophilicity of codrugs **1–3** was also calculated using the ACD Log*P* software package, version 4.55 (Advanced Chemistry Development Inc., Toronto, Canada) (Table 1); as expected, the log*K* value decreased linearly with increasing concentration of acetronitrile in the mobile phase (Fig 2). Stability studies of the new compounds were performed at 37°C in isotonic sodium phosphate buffer (pH = 7.4), in simulated gastric fluid (SGF, pH = 1.3), in rat and human plasma diluted to 80% with isotonic sodium phosphate buffer (pH = 7.4), and in rat brain homogenate as previously described [18]. The disappearance of the codrugs was monitored by the HPLC UV-DAD method and the pseudo-first-order hydrolysis rate constants (K_{obs}) for the hydrolysis were calculated from the slopes of linear plots of the logarithm of residual codrugs **1–3** against time [19]. In buffer solutions, the new compounds were extremely stable in all media ($t_{1/2}$: >85 h for all tested compounds, data not shown). In rat and human plasma, a slow bioconversion to IBU and LA was observed using liquid chromatography/mass spectrometry (LC-MS); we were only able to measure the relative amount of parent drugs. The extracts were analyzed using a LC-MS/MS method previously proposed [20–22] and all MS experiments were performed using electrospray ionization (ESI) mode. Analysis of metabolite by exact mass demonstrated two-peak transitions of *m*/*z*: 205.0 to 171.0 for LA (peak of *m*/*z*: 171 was [LA-H_2S-H]$^-$) and *m*/*z*: 205 to *m*/*z*: 161 for IBU in the negative-ion mode. K_{obs} and the corresponding half-life times ($t_{1/2}$) are shown in Table 2; all the codrugs were extremely stable in human serum, with half-lives exceeding 115 min, indicating high resistance to peripheral enzymatic degradation. The stability of new codrugs towards central enzymatic degradation was also investigated by measuring their bioconversion rates in the presence of rat brain homogenate [23]. The *in-vitro* brain metabolic stabilities are summarized in Table 2; codrugs **1–3** were hydrolyzed more rapidly in brain tissue than in rat serum ($t_{1/2}$ = 17 and 61 min, respectively for compound **1**) indicating that these new entities might allow targeted delivery of the parent drugs to neurons, where cellular oxidative stress and inflammation seem related to AD.

A pseudo-first order degradation of the codrugs **1–3** in brain homogenate was monitored by LC-MS/MS and NMR. The determination of LA, IBU, and their metabolites was based on HPLC coupled with an amperometric electrode-array detector (AEAD) and diode-array detection (DAD), respectively (Figs. 3 and 4). An aliquot (2–3 mg) of the HPLC-purified metabolite was collected, dis-

Table 2. Rate constants for the enzymatic hydrolysis of codrugs **1–3** in 80% rat and human plasma at 37°C and in rat brain homogenate.

Compound	Rat plasma[§]		Human plasma[§]		Brain homogenate[§]	
	$t_{1/2}$ (min)	K_{obs} (min^{-1})	$t_{1/2}$ (min)	K_{obs} (min^{-1})	$t_{1/2}$ (min)	K_{obs} (min^{-1})
1	61.2 ± 1.4	(11.02 ± 0.32) × 10^{-3}	180.3 ± 8.1	(3.84 ± 0.17) × 10^{-3}	17.2 ± 0.6	(40.30 ± 1.41) × 10^{-3}
2	50.5 ± 1.5	(14.07 ± 0.41) × 10^{-3}	121.2 ± 3.4	(5.77 ± 0.14) × 10^{-3}	13.8 ± 0.3	(50.23 ± 1.09) × 10^{-3}
3	43.1 ± 1.1	(16.13 ± 0.40) × 10^{-3}	115.3 ± 4.2	(6.01 ± 0.22) × 10^{-3}	13.4 ± 0.3	(51.73 ± 1.16) × 10^{-3}

§ Values are means of three experiments ± S.D.

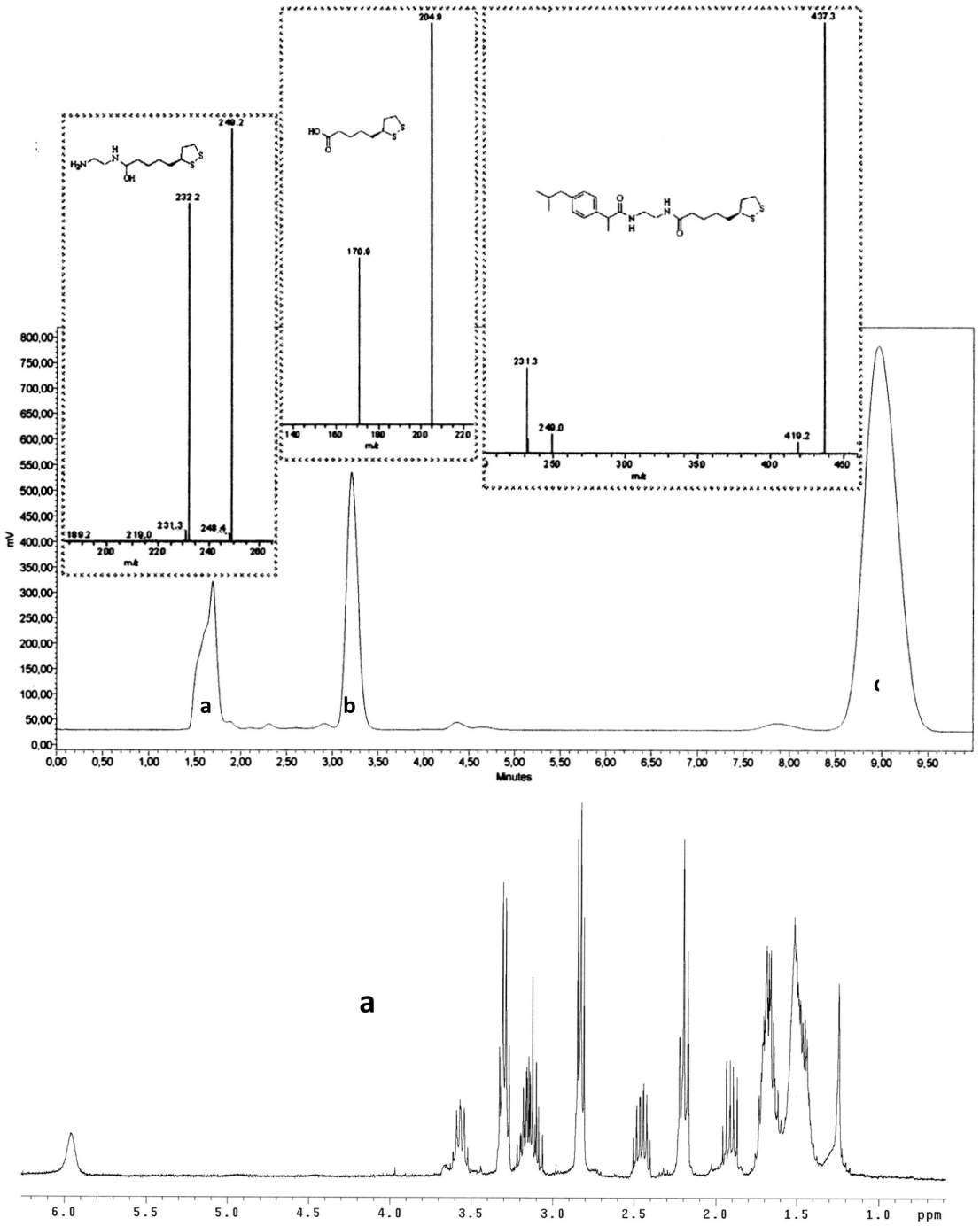


Figure 3. HPLC-AEAD chromatogram, MS and ^{1}H-NMR spectra of codrug **1**, and its LA metabolites in brain homogenate.

solved in DMSO-d_6 (4 mL) and analyzed by ^{1}H- and ^{13}C-NMR. The obtained NMR spectrum indicated that compounds **1**–**3** were converted to IBU and LA and that the diaminoalkyl spacers have little influence on rates of codrugs bioconversion. The reaction seems to proceed by a two-steps reaction in which K_1 is extremely larger than K_2; two alternative biodegradation routes of compound **1** are outlined in Fig. 5. During the hydrolysis of the diaminoalkyl spacers, the amidic residue remains either with LA or IBU. Unfortunately, the direct quantitation of the

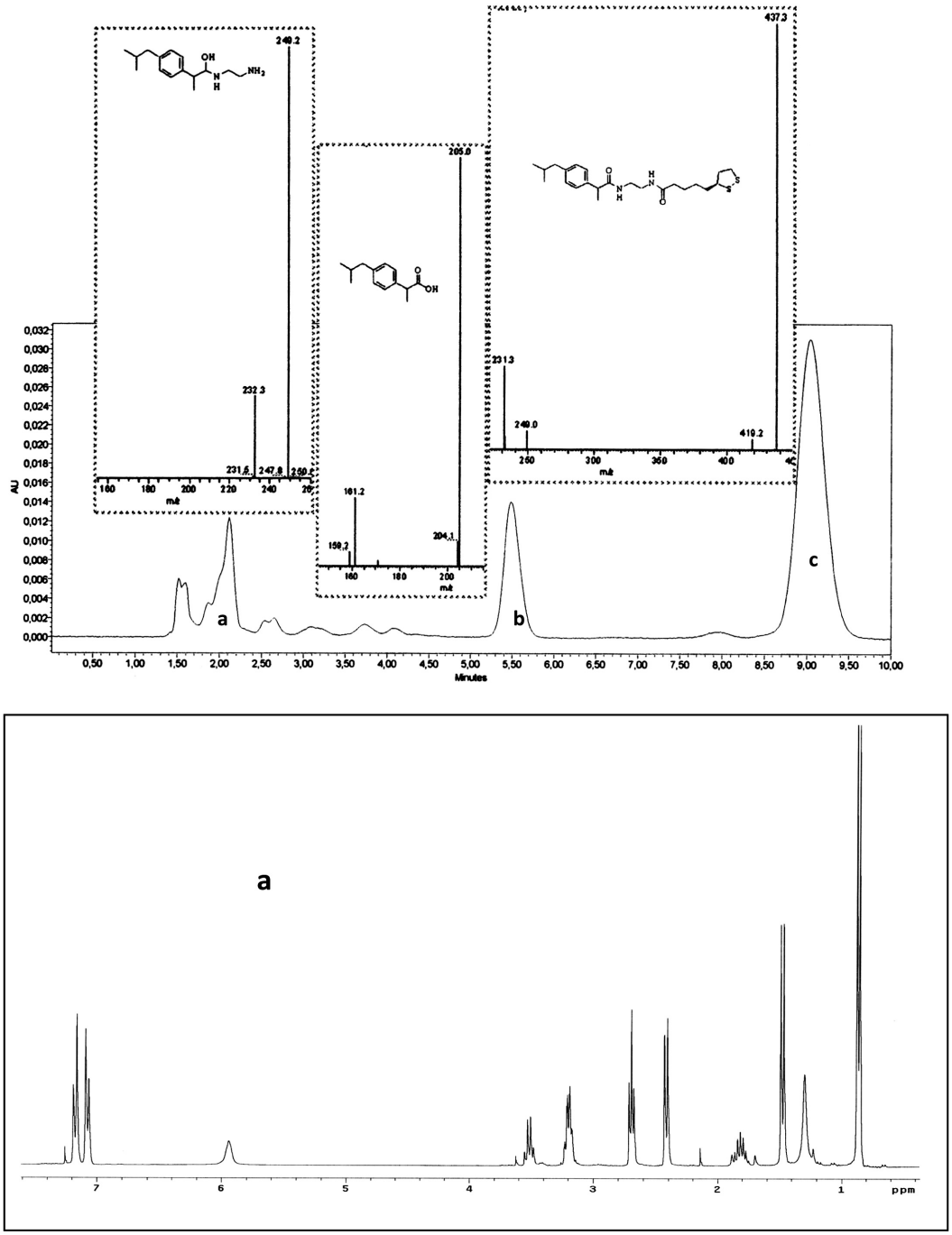


Figure 4. HPLC-DAD chromatogram, MS and ^{1}H-NMR spectra of codrug **1**, and its IBU methabolites in brain homogenate.

intermediates was difficult, due to the high value of K_1 and the consequent rapid bioconversion to the parent drugs.

Two different assays, the rapid DPPH-HPLC method and the deoxyribose oxidation method (DOM), were used to evaluate the free-radical scavenging activity of our compounds. If the tested compound is a free-radical scavenger, it reacts with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and converts it to the stable diamagnetic molecule (1,1-diphenyl-picrylhydrazine). The DPPH has a characteristic absorption at 517 nm: when its electron becomes paired off, the absorption decreases stoichio-

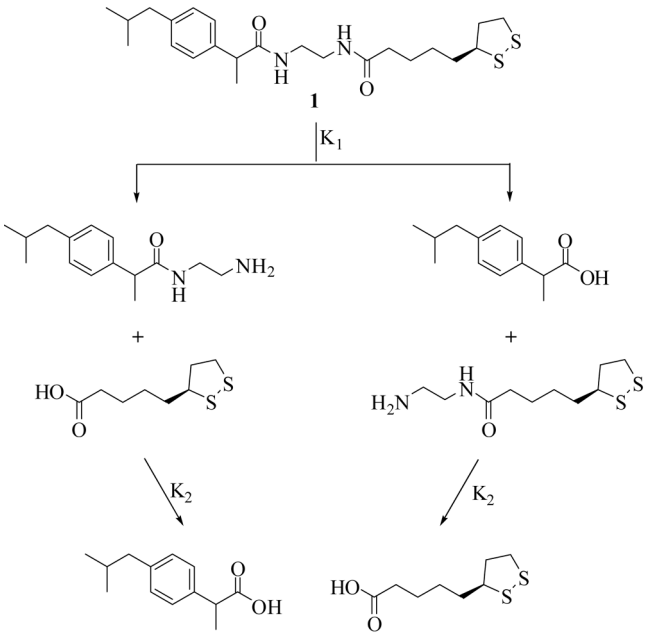


Figure 5. Schematic representation for bioconversion of compound **1**.

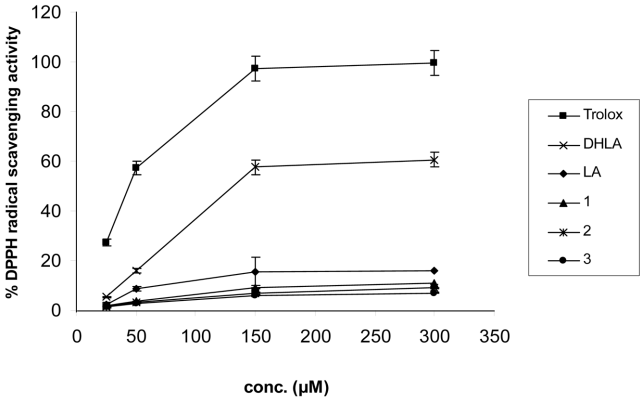


Figure 6. %-DPPH radical-scavenging activity after 20 min of incubation.

metrically with respect to the number of electrons taken up. Since the decrease in the peak depends on the radical-scavenging activity of tested compounds and the time of incubation, the absorbance was recorded at concentrations between 50 and 300 μM and at different incubation times (up to 120 h) [24]. Our results indicated that IBU did not interact with DPPH; instead, the radical-scavenging activity of other tested compounds increased with the concentration (Fig. 6) and with incubation time (data not shown). Moreover, DHLA was stronger than LA and codrugs **1–3** were stronger than IBU, probably because of its dithiol group [25].

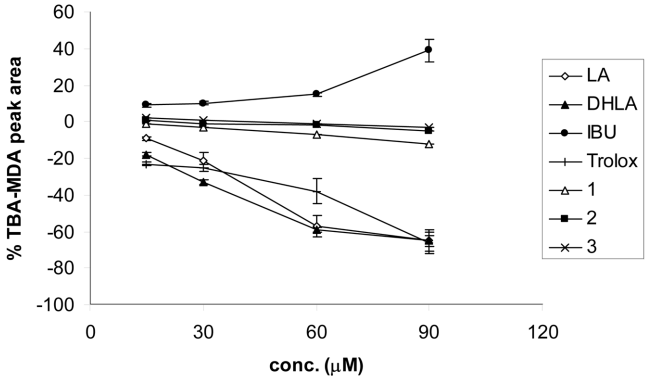
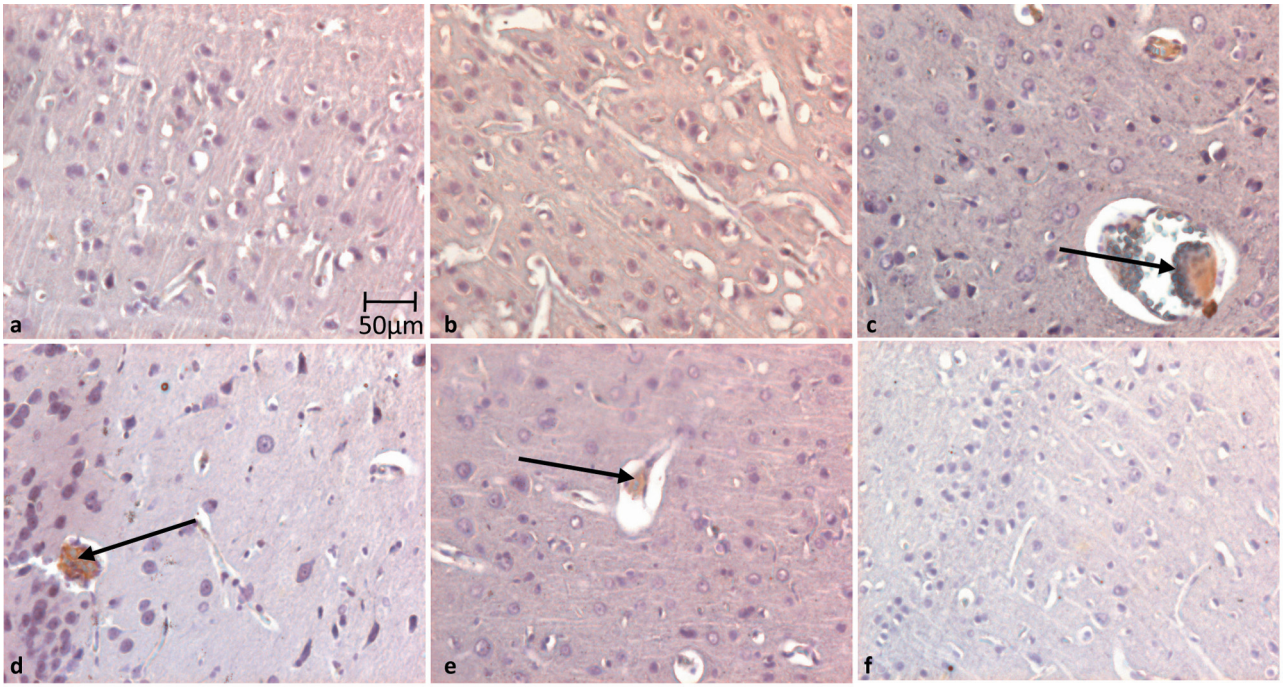


Figure 7- %-TBA-MDA peak area after 20 min of incubation.

The DOM was based on reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA), generated from the oxidative attack of •OH on deoxyribose. In our study, we applied the highly specific and sensitive HPLC method for evaluating the •OH-radical scavenging activity of the synthesized codrugs [26]. The TBA-MDA complex was monitored at 532 nm and, as expected, the peak area decreased in the presence of antioxidant compounds. In our experimental conditions, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, (trolox), LA, and DHLA were antioxidants, while IBU had a pro-oxidant activity (Fig. 7), according to literature data [27, 28]. As expected, codrugs **1–3** were weak antioxidant compounds because the pro-oxidant activity of the IBU moiety was modulated by the dithiol group of LA.

In the pharmacological study, immunohistochemical analysis of Aβ (1-40) protein, which accumulated within capillary vessels, disclosed many different-sized plaques within capillary vessels in the cerebral cortex of Aβ (1-40) and/or drug-vehicle injected rats (Fig. 8). Aβ-injected cerebral cortices treated with IBU or compound **1** showed few plaques within the capillary vessels and, in particular, Aβ (1-40) was less expressed in codrug-**1**-treated than in IBU-treated cerebral cortex.

In summary, this report describes a series of IBU and LA diamides as potential neuroprotective agents in AD therapy. Our findings showed that the new codrugs are characterized by great stability toward rat and human plasma enzymatic activity whereas they were hydrolyzed more rapidly in brain tissue. Codrugs **1–3** displayed free-radical scavenging effects in two different *in-vitro* assay methods (DPPH and DOM) and are able to antagonize the deleterious structural effects in an Aβ-infused AD rat model. Thus, the studied codrugs are promising drug candidates in pathological events in which free-radical damage and inflammatory activity in the brain are involved.



Magnification 206. (**a**): control; (**b**): DMSO/drug vehicle injected cerebral cortex; (**c**): Aβ-injected cerebral cortex; (**d**): Aβ-injected cerebral cortex + IBU; (**e**): Aβ-injected cerebral cortex + **1**; (**f**): negative control (without Aβ (1-40) antibody treatment).
Arrows indicate Aβ plaques in Aβ-injected cerebral cortices, in Aβ-injected cerebral cortices + IBU, and in Aβ-injected cerebral cortices + **1**.

Figure 8. Immunohistochemical detection of Aβ (1-40) in rat brain in different experimental conditions.

Experimental

General

Microanalyses were performed on a 1106 Carlo Erba CHN analyzer (Carlo Erba, Milan, Italy), with results within ± 0.4% of the calculated values.

Codrugs **1–3** were characterized by ^{1}H-, ^{13}C-NMR, and LC-MS, and their purities (>95%) were quantified by HPLC. Analytical HPLC measurements were run on a Waters 1525 Binary HPLC pump (Waters Corporation, Milford, MA, USA), equipped with a Waters 2996 photodiode array detector and an Antec Leyden electrochemical detector with glassy carbon working electrode, a 20-µL Rheodyne injector and a computer-integrating apparatus. The column was a Waters Symmetry RP-C_{18} column (4.6 × 150 mm, 5 µm), the mobile phase was a mixture of 0.05 M KH_2PO_4/acetonitrile (25:75) at a flow rate of 1 mL/min, the UV-detector was set at a length of 264 nm, and the working electrode was set at a potential of +1.1 V vs. Ag/AgCl.

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR 300-MHz spectrometer (Varian Inc., Palo Alto, CA, USA). Chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me_4Si). The LC-MS/MS system employed consisted of an LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. The capillary temperature was set at 300°C and the spray voltage at 4.25 kV. The fluid was nebulized using nitrogen (N_2) as both the sheath gas and the auxiliary gas. The identity of all new compounds was confirmed by elemental analysis, NMR data, and LC-MS/MS system; homogeneity was confirmed by TLC on silica gel Merck 60 F_{254} (Merck, Germany). Solutions were routinely dried over anhydrous sodium sulphate prior to evaporation. Chromatographic purifications were performed by Merck 60 70-230 mesh ASTM silica gel column. IBU, ethylendiamine, 1,4-diaminobutane, 1,6-diaminohexane, LA, acetonitrile, trifluoroacetic acid (TFA), 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDCI), and hydroxybenzotriazole (HOBT) were purchased from Sigma Aldrich.

Chemistry

Ibuprofen methylester **4** was synthesized as previously reported [29]. Compounds **1–3** were synthesized as outlined in Scheme 1. Aminoamides **5–7** were synthesized by heating ibuprofen methylester **4** with ethylendiamine, 1,4-diaminobutane, and 1,6-diaminohexane, respectively, then, lipoic acid was coupled to aminoamides **5–7** using HOBT and EDCI as peptide-coupling agents [30].

*General procedure for the preparation of the aminoamides **5–7***

A mixture of ibuprofen methylester (870 mg, 4.0 mmol) and 105.0 mmol of alkyldiamine was heated at 110°C for 4 h. The reaction mixture was diluted with $CHCl_3$ and washed with saturated aqueous $NaHCO_3$. The organic layer was dried and the solvent was evaporated *in vacuo*.

*N-(2-Aminoethyl)-2-(4-isobutylphenyl)propanamide **5***

Yield: 66%; R_f = 0.18, $CHCl_3$:MeOH (6:4); ^{1}H-NMR ($CDCl_3$) δ: 7.19 (d, 2H, *J* = 8.0 Hz, ArH), 7.09 (d, 2H, *J* = 8.0 Hz, ArH), 5.98 (m, 1H, NH),

3.53 (dd, 1H, J_1 = 7.2 Hz, J_2 = 15.0 Hz, CH), 3.25–3.18 (m, 2H, CH_2), 2.70 (t, 2H, J = 5.7 Hz, CH_2), 2.43 (d, 2H, J = 7.2 Hz, CH_2), 1.84–1.80 (m, 1H, CH), 1.78 (m, 2H, NH_2), 1.49 (d, 3H, J = 7.2 Hz, CH_3), 0.88 (d, 6H, J = 6.6 Hz, 2 CH_3); ^{13}C-NMR ($CDCl_3$) δ: 175.13 (s, 1C, CO), 140.89 (s, 1C, Ar), 138.94 (s, 1C, Ar), 129.82 (s, 2C, Ar), 127.49 (s, 2C, Ar), 46.94 (s, 1C, CH), 45.22 (s, 1C, CH_2), 42.15 (s, 1C, CH_2), 41.42 (s, 1C, CH_2), 30.41 (s, 1C, CH), 22.60 (s, 2C, CH_3), 18.77 (s, 1C, CH_3). Anal. ($C_{15}H_{24}N_2O$) C, H, N.

N-(4-Aminobutyl)-2-(4-isobutylphenyl)propanamide ***6***

Yield: 54%; R_f = 0.20, $CHCl_3$: MeOH (6:4); ^{1}H-NMR ($CDCl_3$) δ: 7.16 (d, 2H, J = 8.10 Hz, ArH), 7.07 (d, 2H, J = 8.10 Hz, ArH), 5.97 (m, 1H, NH), 3.50 (dd, 1H, J_1 = 7.2 Hz, J_2 = 14.4 Hz, CH), 3.21–3.09 (m, 2H, CH_2), 2.54–2.48 (m, 2H, CH_2), 2.61 (m, 2H, NH_2), 2.42 (d, 2H, J = 7.2 Hz, CH_2), 1.86–1.76 (m, 1H, CH), 1.46 (d, 3H, J = 7.2 Hz, CH_3), 1.40–1.34 (m, 4H, 2 CH_2), 0.86 (d, 6H, J = 6.6 Hz, 2 CH_3); ^{13}C-NMR ($CDCl_3$) δ: 174.69 (s, 1C, CO), 140.80 (s, 1C, Ar), 138.99 (s, 1C, Ar), 129.74 (s, 2C, Ar), 127.58 (s, 2C, Ar), 46.92 (s, 1C, CH), 45.19 (s, 1C, CH_2), 41.81 (s, 1C, CH_2), 39.61 (s, 1C, CH_2), 30.90 (s, 1C, CH), 30.40 (s, 1C, CH_2), 27.11 (s, 1C, CH_2), 22.58 (s, 2C, CH_3), 18.71 (s, 1C, CH_3). Anal. ($C_{17}H_{28}N_2O$) C, H, N.

N-(6-Aminohexyl)-2-(4-isobutylphenyl)propanamide ***7***

Yield: 62%; R_f = 0.22, $CHCl_3$:MeOH (6:4); ^{1}H-NMR ($CDCl_3$) δ: 7.20 (d, 2H, J = 8.1 Hz, ArH), 7.08 (d, 2H, J = 8.1 Hz, ArH), 6.01 (m, 1H, NH), 3.56 (dd, 1H, J_1 = 7.2 Hz, J_2 = 14.4 Hz, CH), 3.47 (m, 2H, NH_2), 3.17–3.07 (m, 2H, CH_2), 2.96–2.92 (m, 2H, CH_2), 2.43 (d, 2H, J = 7.2 Hz, CH_2), 1.87–1.78 (m, 1H, CH), 1.74–1.69 (m, 2H, CH_2), 1.46 (d, 3H, J = 7.2 Hz, CH_3), 1.42–1.34 (m, 4H, 2 CH_2), 1.26–1.21 (m, 2H, CH_2), 0.87 (d, 6H, J = 6.6 Hz, 2 CH_3); ^{13}C-NMR ($CDCl_3$) δ: 175.21 (s, 1C, CO), 140.88 (s, 1C, Ar), 138.79 (s, 1C, Ar), 129.78 (s, 2C, Ar), 127.56 (s, 2C, Ar), 46.73 (s, 1C, CH), 45.21 (s, 1C, CH_2), 39.86 (s, 1C, CH_2), 39.38 (s, 1C, CH_2), 30.41 (s, 1C, CH), 29.14 (s, 1C, CH_2), 27.48 (s, 1C, CH_2), 25.94 (s, 1C, CH_2), 25.76 (s, 1C, CH_2), 22.62 (s, 2C, CH_3), 18.76 (s, 1C, CH_3). Anal. ($C_{19}H_{32}N_2O$) C, H, N.

General procedure for the preparation of codrugs ***1–3***

Compound **5–7** (8.81 mmol), TEA (1.6 mL), HOBT (1.70 g, 12.6 mmol), EDCI (4.82 g, 25.2 mmol), and additional TEA (1.6 mL) were added successively to a solution of lipoic acid (2.00 g, 9.694 mmol) in dichloromethane (40 mL). The reaction mixture was stirred overnight at 25°C and diluted with water (100 mL). The product was extracted with dichloromethane (3 6 100 mL), dried, filtered, and then evaporated *in vacuo* to give a residue which was purified by column chromatography with $CHCl_3$/MeOH (95:5) as eluent.

5-(1,2-Dithiolan-3-yl)-N-(2-{[2-(4-isobutylphenyl)propanoyl]amino}ethyl)pentanamide ***1***

Yield: 50%; R_f = 0.24, $CHCl_3$: MeOH (95:5); ^{1}H-NMR ($CDCl_3$) δ: 7.18 (d, 2H, J = 8.10 Hz, ArH), 7.10 (d, 2H, J = 8.10 Hz, ArH), 6.18 (m, 1H, NH), 5.98 (m, 1H, NH), 3.58–3.49 (m, 2H, 2 CH), 3.35–3.22 (m, 4H, 2 CH_2), 3.18–3.09 (m, 2H, CH_2), 2.49–2.43 (m, 3H, CH and CH_2), 2.10 (t, 2H, J = 7.4 Hz, CH_2), 1.93–1.82 (m, 2H, CH_2), 1.72–1.58 (m, 4H, 2 CH_2), 1.49 (d, 3H, J = 7.2 Hz, CH_3), 1.47–1.39 (m, 2H, CH_2), 0.89 (d, 6H, J = 6.6 Hz, 2 CH_3); ^{13}C-NMR ($CDCl_3$) δ: 176.23 (s, 1C, CO), 174.07 (s, 1C, CO), 140.98 (s, 1C, Ar), 138.58 (s, 1C, Ar), 129.81 (s, 2C, Ar), 127.44 (s, 2C, Ar), 56.66 (s, 1C, CH), 46.82 (s, 1C, CH), 45.23 (s, 1C, CH_2), 40.48 (s, 1C, CH_2), 40.30 (s, 1C, CH_2), 40.10 (s, 1C, CH_2), 38.71 (s, 1C, CH_2), 36.47 (s, 1C, CH_2), 34.86 (s, 1C, CH_2), 30.42 (s, 1C, CH), 29.14 (s, 1C, CH_2), 25.57 (s, 1C, CH_2), 22.64 (s, 2C, CH_3), 18.68 (s, 1C, CH_3); MS (ESI) m/z: 437 [M^+ + H]. Anal. ($C_{23}H_{36}N_2O_2S_2$) C, H, N, S.

5-(1,2-Dithiolan-3-yl)-N-(4-{[2-(4-isobutylphenyl)propanoyl]amino}butyl)pentanamide ***2***

Yield: 60%; R_f = 0.26, $CHCl_3$:MeOH (95:5); ^{1}H-NMR ($CDCl_3$) δ: 7.19 (d, 2H, J = 8.0 Hz, ArH), 7.08 (d, 2H, J = 8.0 Hz, ArH), 5.76 (m, 1H, NH), 5.55 (m, 1H, NH), 3.52–3.49 (m, 2H, 2 CH), 3.41–3.39 (m, 4H, 2 CH_2), 3.24–3.11 (m, 2H, CH_2), 2.46–2.42 (m, 3H, CH and CH_2), 2.15 (t, 2H, J = 7.2 Hz, CH_2), 1.95–1.84 (m, 2H, CH_2), 1.70–1.62 (m, 6H, 3 CH_2), 1.50 (d, 3H, J = 7.2 Hz, CH_3), 1.47–1.40 (m, 4H, 2 CH_2), 0.88 (d, 6H, J = 6.6 Hz, 2 CH_3); ^{13}C-NMR ($CDCl_3$) δ: 174.98 (s, 1C, CO), 173.06 (s, 1C, CO), 140.98 (s, 1C, Ar), 138.78 (s, 1C, Ar), 129.86 (s, 2C, Ar), 127.58 (s, 2C, Ar), 56.69 (s, 1C, CH), 46.99 (s, 1C, CH), 45.23 (s, 1C, CH_2), 40.48 (s, 1C, CH_2), 39.29 (s, 1C, CH_2), 39.19 (s, 1C, CH_2), 38.71 (s, 1C, CH_2), 36.71 (s, 1C, CH_2), 34.86 (s, 1C, CH_2), 30.43 (s, 1C, CH), 29.15 (s, 1C, CH_2), 27.25 (s, 1C, CH_2), 26.80 (s, 1C, CH_2), 25.65 (s, 1C, CH_2), 22.62 (s, 2C, CH_3), 18.72 (s, 1C, CH_3); MS (ESI) m/z: 465 [M^+ + H]. Anal. ($C_{25}H_{40}N_2O_2S_2$) C, H, N, S.

5-(1,2-Dithiolan-3-yl)-N-(6-{[2-(4-isobutylphenyl)propanoyl]amino}hexyl)pentanamide ***3***

Yield: 55%; R_f = 0.27, $CHCl_3$:MeOH (95:5); ^{1}H-NMR ($CDCl_3$) δ: 7.18 (d, 2H, J = 8.1 Hz, ArH), 7.10 (d, 2H, J = 8.1 Hz, ArH), 5.67 (m, 1H, NH), 5.43 (m, 1H, NH), 3.57–3.51 (m, 2H, 2 CH), 3.18–3.05 (m, 6H, 3 CH_2), 2.50–2.41 (m, 3H, CH and CH_2), 2.17 (t, 2H, J = 7.4 Hz, CH_2), 1.95–1.80 (m, 2H, CH_2), 1.69–1.62 (m, 6H, 3 CH_2), 1.49 (d, 3H, J = 7.2 Hz, CH_3), 1.47–1.34 (m, 6H, 3 CH_2), 1.30–1.19 (m, 2H, CH_2), 0.89 (d, 6H, J = 6.6 Hz, 2 CH_3); ^{13}C-NMR ($CDCl_3$) δ: 174.80 (s, 1C, CO), 172.95 (s, 1C, CO), 140.94 (s, 1C, Ar), 138.84 (s, 1C, Ar), 129.83 (s, 2C, Ar), 127.57 (s, 2C, Ar), 56.70 (s, 1C, CH), 47.02 (s, 1C, CH), 45.22 (s, 1C, CH_2), 41.02 (s, 1C, CH_2), 40.48 (s, 1C, CH_2), 39.18 (s, 1C, CH_2), 39.09 (s, 1C, CH_2), 38.70 (s, 1C, CH_2), 36.76 (s, 1C, CH_2), 34.86 (s, 1C, CH_2), 30.42 (s, 1C, CH), 29.59 (s, 1C, CH_2), 29.16 (s, 1C, CH_2), 26.07 (s, 1C, CH_2), 25.94 (s, 1C, CH_2), 25.70 (s, 1C, CH_2), 22.61 (s, 2C, CH_3), 18.70 (s, 1C, CH_3); MS (ESI) m/z: 493 [M^+ + H]. Anal. ($C_{27}H_{44}N_2O_2S_2$) C, H, N, S.

Aqueous solubility

Compounds **1–3** (50 mg) were placed in a microtube containing 1 mL of deionized water and were shaken at 25°C for 1 h to ensure the solubility equilibrium. After centrifugation, a 20-μL portion of the supernatant was analyzed by HPLC [31].

Lipophilicity

clogP

The calculated clogP was determined using ACD LogP software package, version 4.55 (Advanced Chemistry Development Inc., Toronto, Canada).

Logk_0

Oil/water partition coefficients can be estimated using RT due to the good relationship between log octanol/water partition coefficients and log*k* values determined using octadecyl silica columns. Each compound was dissolved in methanol (concentration 1 mg/mL). Aliquots of each solution were filtered and analyzed by the HPLC. The mobile phase consisted of acetonitrile and water with acetonitrile content between 90 and 55% (v/v) in 5% increments [32]. The dead time was measured by injection of

methanol as a non-retained compound. The isocratic capacity factors log*k*, were calculated from the dead-times (t_0) and retention-times (t_r) values by use of the equation:

$$\log k_0 = \log\left[\frac{t_r - t_0}{t_0}\right] \quad (1)$$

The $\log k_0$ values were extrapolated to zero-acetonitrile content by linear plots of acetonitrile concentration against log*k*.

Kinetics of chemical hydrolysis

5 mg of codrugs **1–3** were dissolved in 50 μL of DMSO and added to a solution of 70 mL of 0.02 M buffer (pH = 1.3, 5.0, and 7.4) and 30 mL of acetonitrile. The resulting mixture was sonicated for 3 min, and then stirred at 37 ± 0.5°C. At different intervals of time, an aliquot (20 μL) of this solution was collected and analyzed by HPLC [33].

Kinetics of enzymatic hydrolysis

The enzymatic hydrolysis of compounds **1–3** was evaluated in rat plasma at 37°C. Stock solutions were prepared by dissolving 5 mg of codrug in 50 μL of DMSO. This solution was added with 4 mL of pre-warmed (37°C) plasma previously diluted to 80% with 50 mM phosphate buffer, pH = 7.4, pre-thermostated at 37°C. The resulting solution was kept at 37°C and 0.2 mL samples were withdrawn at intervals and added to 0.4 mL of cold (4°C) acetonitrile to precipitate serum proteins. After centrifugation for 10 min at 10 000 rpm and at 5°C, the supernatant was assayed by the HPLC method previously described [34].

Degradation by brain homogenate

The degradation studies were performed on the rat brain homogenate, according to the modified method described by Perlikowska *et al*. [23]. Briefly, rat brains were isolated, pooled, homogenized with 20 volumes of 50 mM Tris-HCl (pH = 7.4), and stored at −80°C until used. The aliquots (100 μL, 10 mg protein/mL) were incubated with 100 μL of compound (0.5 mM) over 0, 7.5, 15, 22.5, 30, and 60 min at 37°C in a final volume of 200 μL. The reaction was stopped at the required time by placing the tube on ice and acidifying with 20 μL of 1 M aqueous HCl solution. The aliquots were centrifuged at 20 000 × *g* for 10 min at 4°C. The obtained supernatants were filtered and analyzed by HPLC.

DPPH-HPLC method

The DPPH radical scavenging activity was estimated according to the method explained by Chandrasekar *et al*. (2006) with some modifications [35]. A stock solution of 1 mM DPPH in methanol was prepared. 200 μL of tested compounds (25–300 μM in methanol) were added to 200 μL of DPPH stock solution and the mixture was vortexed for a few seconds and left to stand in the dark for 20 min at room temperature. The blank was prepared by adding 200 μL of methanol to 200 μL of DPPH stock solution. Trolox was used as control standard [36]. All solutions were filtered and analyzed by HPLC. In our HPLC conditions, the retention time of the DPPH peak is 5.82 min.

The DPPH peak was monitored at 517 nm and the radical-scavenging activity of each sample was calculated from the peak area (PA) as reported above:

$$\text{Radical scavening } (\%) = \left[\frac{\text{PA}_{\text{blank}} - \text{PA}_{\text{sample}}}{\text{PA}_{\text{blank}}}\right] \times 100 \quad (2)$$

Deoxyribose-oxidation method (DOM)

The ·OH-radical scavenging activity of codrugs was analyzed by the Fe(II)/H_2O_2-induced degradation of deoxyribose assay conducted in the presence of EDTA as previously described with some modification [37]. Briefly, the reaction mixtures contained, in a final volume of 300 μL, the following reagents at the final concentrations stated: deoxyribose (28 mM), potassium phosphate buffer, pH = 7.4 (100 mM), increasing concentrations of compounds **1–3** (15–20 μM), $(NH_4)_2Fe(SO_4)_2$ (0.1 mM), EDTA (0.2 mM), and H_2O_2 (10 mM). The reactions were carried out at 37°C for 30 min; then 300 μL of 1% (w/v) thiobarbituric acid (TBA) in 50 mM NaOH and 300 μL of 2.8% (w/v) trichloroacetic acid were added. After heating at 100°C for 30 min, the reaction solutions were cooled and centrifugated at 14 000 × *g* [38]. The supernatant was analyzed by HPLC using as mobile phase 50 mM KH_2PO_4 buffer solution, methanol, and acetonitrile (72:17:11) [39]. In our HPLC conditions, the retention time of TBA-MDA peak is 2.70 min. Trolox was used as control standard.

Pharmacological procedures

Animals

Male Wistar rats (Harlan, UD, Italy) that weighed 200 to 225 g at the beginning of the experiments were used. All procedures were conducted in agreement with the European Community Council Directive for Care and Use of Laboratory Animals.

Surgical procedure

The rats were anesthetized with a mixture of zolazepam and tiletamine (10 mg/kg, i.p.). Aβ (1-40) was dissolved in sterile saline containing 35% acetonitrile and 0.1% TFA. Continuous infusion of Aβ (1-40) solution (4.6 nmol/rat at a final volume of 200 μL) or the vehicle alone was delivered for 28 days by attachment of an infusion kit connected to an osmotic pump (Alzet model 2004, Charles River, Italy). The infusion kit was implanted into the right ventricle (1.0 mm posterior to the bregma, 1.8 mm lateral to the midline, and 3.5 mm ventral to the surface of the skull, according to the brain atlas of Paxinos and Watson (1986).

Drug administration

IBU and **1** were both solubilized in sterile saline containing 20% (v/v) DMSO and were daily administered subcutaneously (s.c.) for 28 days at a dose of 5 mg/kg and 10 mg/kg, respectively. A vehicle solution (vehicle for s.c. injections) prepared with sterile saline containing 20% (v/v) DMSO or a sterile saline alone, were also administered s.c. for 28 days at a dose volume of 250 μL/kg as IBU and **1**.

Immunohistochemical analysis

Two months after surgery, rats were sacrified with anhydride carboxide and their whole brains were removed for immunohistochemical analysis. Rat brains were fixed in 10% (v/v) phosphate-buffered fomalin and then paraffin embedded. The samples were then de-waxed (xylene and alcohol progressively lower concentrations) and processed. Brain sections (5 μm) were first blocked in 5% normal goat serum (NGS) and then incubated in the presence of mouse anti-human Aβ (1-40) monoclonal antibody (Li StarFish, Milano, Italy) and in the presence of HRP-conjugated secondary antibody. Immunohistochemistry was performed using an UltraVision LP Detection System HRP Polymer & DAB Plus Chromogen kit (Thermo Fisher Scientific, CA, USA) and

processed according to data sheet. Peroxidase was developed using diaminobenzidine chromogen (DAB) (Biomeda Corp., CA, USA) and nuclei were hematoxylin counterstained. Negative controls were performed by omitting the primary antibody. Samples were then observed with a light microscope (Leica, Heidelberg, Germany) equipped with a Coolsnap video camera for computerized images (RS Photometrics, Tucson, AZ, USA).

Computerized morphometry measurements and image analysis

After digitizing the images deriving from immunohistochemistry stained sections, Metamorph Software System (Universal Imaging Corporation, Molecular Device Corporation, PA, USA) (Crysel Instruments, Rome, Italy) was used to evaluate Aβ (1-40) expression. Image analysis of protein expression was performed through the quantification of the tresholded area for immunohistochemical brown colors per field of light microscope observation.

Metamorph assessments were logged to Microsoft Excel and processed for standard deviations and histograms.

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