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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 825–829

Synthesis and pharmacochemical study of novel polyfunctional molecules combining anti-inflammatory, antioxidant, and hypocholesterolemic properties

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Received 24 September 2005; revised 5 November 2005; accepted 7 November 2005
Available online 23 November 2005

Abstract—We have designed and synthesized a series of novel molecules having a residue of a classical NSAID and an antioxidant moiety, both attached through amide bonds to a known nootropic structure, an L-proline, *trans*-4-hydroxy-L-proline or DL-pipecolinic acid residue. The compounds were found to retain anti-inflammatory and antioxidant activities, to acquire hypocholesterolemic action, and to possess a greatly reduced gastrointestinal toxicity. The novel molecules could find useful applications, among others, in slowing the progression or delaying the onset of neurodegenerative diseases.

Alzheimer's disease and related dementias (SDAT) are the greatest unmet medical challenges in neurology, with over 12 million sufferers in the world. This condition accounts for the majority of dementias diagnosed over the age of 60. It is characterized by a global, progressive decline of cognitive functions and leaves end-stage patients bedridden, dependent on custodial care, with death occurring in about 10 years after diagnosis.[1] Pharmacotherapy of SDAT has been based on the cholinergic hypothesis, that is, the dysfunction of the acetylcholine system contributes to cognitive derangement in SDAT. Therefore, standard care includes treatment with acetylcholinesterase inhibitors.[2] These agents have been proven of limited benefit, while this symptomatic treatment fails to inhibit the progress of the disease itself.

It is well documented that inflammation[3] as well as oxidative stress[4,5] are profoundly implicated in a number of pathobiochemical processes related to neurodegenerative diseases. Furthermore, increased plasma cholesterol is related to neurodegeneration.[6] Thus, the prevention of these biochemical aberrations occurring in the demented brain may be a more rational approach toward agents against this pathological condition. In this investigation, we have designed a series of novel polyfunctional molecules combining anti-inflammatory, antioxidant, and hypocholesterolemic properties, which may have the potential to slow or interrupt the progress of the disease and thus provide a more complete and effective treatment approach.

We have recently shown that the chemical derivatization of the carboxylic acid group of well-established nonsteroidal anti-inflammatory drugs (NSAIDs) may offer a viable route to anti-inflammatory agents possessing an increased safety profile.[7] Hence, amidation of the NSAID molecules with cysteamine[8] or cysteine ethyl ester[7] resulted in compounds with increased anti-inflam-

Keywords: Neurodegeneration; L-Cysteine; NSAIDs; Proline amide conjugates; Antioxidants; Anti-inflammatories; Lipoxygenase; Hypocholesterolemics; Gastrointestinal toxicity.
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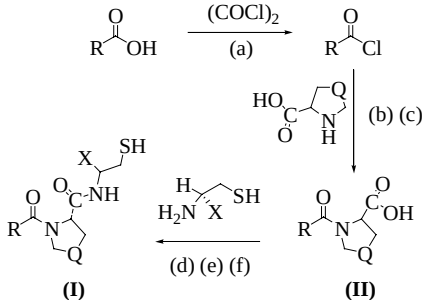
doi:10.1016/j.bmcl.2005.11.027

matory and antioxidant properties but with a significantly lower GI toxicity. Furthermore, it has been reported that a proline moiety is part of the pharmacophore of molecules showing nootropic or antiamnesic activity.[9,10] Thus, we have designed molecules of the general structure **I** (Table 1) in which a NSAID and cysteamine or cysteine ethyl ester have been chemically attached to a proline, 4-hydroxy-proline or pipecolinic acid moiety.

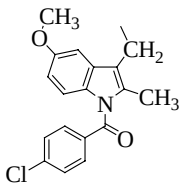
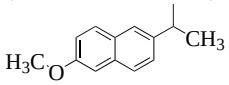
The structures and the synthesis of the novel compounds **1**–**7** are presented in Table 1. They were prepared by two successive amidations, initially of the amine group of the sodium salts of L-proline, *trans*-4-hydroxy-L-proline or DL-pipecolinic acid by the acid chloride of the NSAIDs, and then of the free carboxylic group of the intermediate **II**, using 1,1-carbonyldiimidazol (CDI) under the conditions indicated in Table 1. This group of compounds includes two representative NSAIDs (indomethacin and naproxen) and two known radical scavengers (cysteamine and cysteine ethyl ester). The structures were confirmed spectroscopically (IR, ^{1}H NMR) and by elemental analysis.

Compounds **1**–**7**, along with cysteamine and cysteine ethyl ester as reference compounds, were assayed in vitro for their antioxidant activity by evaluating their ability to inhibit peroxidation of rat hepatic microsomal membrane lipids.[8,11] Four compounds inhibited lipid peroxidation by 100% at 1 mM, while cysteamine and cysteine ethyl ester inhibited lipid peroxidation by 37% and 49%, respectively, at the same concentration. IC_{50} values of the new compounds (incubation for 45 min) were (compound, IC_{50} μM): **2**, 390; **4**, 320; **6**, 500; **7**, 122. This action could be attributed to a combination of proper lipophilicity with the free HS-group which can readily donate the sulfydryl H atom, acting as a chain breaking antioxidant.[12] It is noteworthy that the less lipophilic cysteamine analogues **1**, **3**, and **5** were much less active in this assay ($IC_{50} > 1$ mM) compared with the cysteine ethyl ester analogues **2**, **4**, and **6**. A similar trend was observed in a series of simpler conjugates of NSAIDs with cysteamine[8] and cysteine ethyl ester,[7] the latter being significantly more potent than the former. The parent NSAIDs and the proline intermediates **II** presented negligible antioxidant activity at 1 mM.

Table 1. Synthesis and structures of the prepared compounds



Reagents and conditions: (a) in dichloromethane at 0 °C, 30 min, 3 h rt; (b) in 2 N aq NaOH, 0 °C, 1 h; (c) acidification with 1 N hydrochloric acid, extraction with $CHCl_3$; (d) in DMF, CDI added at 0 °C, 1 h rt; (e) cysteamine HCl or cysteine ethyl ester HCl added at rt and stirred for 24 h; (f) water added, extraction with $CHCl_3$

Compound	R	Q	X
1	 (Indomethacin residue)	CH_2	H
2		CH_2	$COOC_2H_5$
3	 (Naproxen residue)	CH_2	H
4		CH_2	$COOC_2H_5$
5		CHOH	H
6		CHOH	$COOC_2H_5$
7		$(CH_2)_2$	$COOH_2H_5$

Compound, mp (°C) and yield (% final reaction): **1**, 138–140, 29; **2**, 109–111, 30; **3**, 151–152, 42; **4**, oil, 60; **5**, 140–141, 29; **6**, semi-solid, 32; **7**, oil, 39.

The radical scavenging ability of the molecules was determined from the extent of their interaction with the stable free radical DPPH.[13] At equimolar concentrations, the interaction of the compounds with DPPH ranged from 56% to 86% (Table 2). The interaction of the parent NSAIDs with DPPH at the same concentration was negligible. This interaction indicates the reducing potential of the new compounds.

The anti-inflammatory activity of derivatives **1**–**7**, along with indomethacin and naproxen as reference compounds, was assessed from their ability to inhibit paw edema induced by carrageenan in female Fischer rats[7] (Table 3). The compounds were administered ip at a dose of 300 μmol/kg and demonstrated a significant inhibition of the edema ranging from 14% to 68%. Furthermore, experimental arthritis was produced in rats by an id injection of complete Freund's adjuvant.[14] Compounds **1**–**4** were administered at a dose of 300–600 μmol/kg for 14 days, and the arthritic score was assessed on the 15th day post adjuvant injection. The tested compounds inhibited arthritis by 50–100% (Table 4). It can be seen from the results that the new compounds acquire a good antioxidant and reducing potential, while they retain considerable anti-inflammatory activity.

Table 2. Percent interaction of compounds **1**–**7** at various concentrations with DPPH (0.2 mM) after 30 min of incubation

Compound	0.2 mM	0.1 mM	0.05 mM
1	NA[a]	NA[a]	NA[a]
2	69.7	44.2	31.1
3	78.9	56.3	28.8
4	86.3	61.9	32.1
5	56.2	36.5	21.3
6	75.7	54.4	26.8
7	76.7	46.0	23.5

[a] Not active.

Table 3. Anti-inflammatory action of compounds **1**–**7** administered at 300 μmol/kg using the carrageenan paw edema model

Compound	% paw weight increase[a]	% edema inhibition
Control	58.5 ± 4.3	—
Indomethacin	27.0 ± 4.0	53.9**
1	48.5 ± 4.5	17.0**
2	50.1 ± 7.0	14.3*
Naproxen	28.7 ± 4.2	51.0**
3	42.9 ± 11.2	26.7**
4	44.6 ± 8.9	23.7**
5	44.4 ± 9.4	24.1**
6	18.4 ± 2.9	68.5**
7	34.9 ± 2.9[b]	50.7**

[a] Each value is the mean from 4 to 9 rats in two independent experiments. Statistical significance compared with controls.
[b] Compound **7** was tested at a later stage of the project using control group of animals in which the % paw weight increase was 70.8 ± 10.6.
* $P < 0.05$.
** $P < 0.001$ (Student's t test).

Table 4. Effect of the test compounds on Freund's complete adjuvant-induced experimental arthritis in rats

Compound	Arthritic score[a]	% arthritis inhibition
1	7.0 ± 0.8	50.8**
Control	14.3 ± 1.5	—
2	1.8 ± 0.9	77.4*
Control	7.8 ± 2.3	—
3	3.8 ± 1.7	51.6*
Control	7.8 ± 2.3	—
4	0.0 ± 0.0	100**
Control	6.8 ± 2.0	—

[a] Each value is the mean from 4 to 6 rats. Statistical significance compared with controls.
* $P < 0.05$.
** $P < 0.001$ (Student's t test).

It has been reported that lipoxygenases and their metabolic products are increased in the pathologically affected brain regions of Alzheimer's disease (AD) patients.[15] Inflammatory cascade may take part in aging-associated neurodegenerative conditions.[16] Thus, we examined the in vitro effect of three of the synthesized compounds on soybean lipoxygenase [220 U/ml, using sodium linoleate, (100 μM), as substrate, at pH 9 and at 28 °C for 5 min].[17] They exhibited considerable lipoxygenase inhibition, with IC_{50} values (compound, IC_{50} μM): **2**, 200; **3**, 35; **4**, 48.

The gastrointestinal toxicity profiles of compounds **1**–**4** and of the parent NSAIDs were evaluated in vivo following a four-day dosage scheme in rats.[7] Thus, equimolar dosage of the parent NSAIDs, producing ca. 50% mortality in 4 days, and the compounds under investigation were administered sc once daily for four days to female rats. Percent mortality, incidence of perforating gastrointestinal (GI) ulcers, melena defecation, and body weight loss were recorded 24 h after the last treatment (Table 5). Evidently, the new compounds examined are practically devoid of GI toxicity at the doses shown in Table 5, in marked contrast to the parent NSAIDs, that caused 50% mortality and 50–100% GI perforating ulcers. It is known that the formation of gastric lesions by NSAIDs is mainly due to inhibition of prostaglandin biosynthesis. However, the involvement of active oxygen species and lipid peroxidation in the development of NSAID-induced mucosal damage has also been proposed as an important event. Thus, indomethacin administration to rats results in a marked increase of thiobarbituric acid reactive substance formation in the liver and in the induced gastric lesions,[18] while liver glutathione levels are significantly lowered.[19] These findings indicate that NSAIDs increase the susceptibility of tissues to lipid peroxidation[20] and further suggest the potentially important contribution of lipid peroxidation to NSAID-induced ulceration. It is likely that the significantly reduced GI toxicity of the molecules reported here is due to a combination of the antioxidant properties of the compounds and the masking of the free carboxylic group of the parent NSAID. It is noteworthy that HS-containing compounds have been shown to possess healing properties[21], and cysteamine and other HS-derivatives have been reported to exert a protective effect against ethanol- and prostaglandin-inhibition-induced mucosal damage.[22]

The involvement of inflammation[3] and oxidative stress[4,5] in the pathobiochemistry of neurodegenerative diseases is well established. Thus, a number of epidemiological studies show a lower incidence of AD when NSAIDs are taken on a regular basis.[23] In a 15-year longitudinal analysis, the use of NSAIDs was associated with a lower incidence of AD.[24] However, chronic use of NSAIDs in such conditions is seriously limited by their GI-toxicity. Hence, in a double-blind AD treatment trial in which indomethacin was studied with positive results, approximately one-third of the patients had to discontinue because of gastrointestinal side effects.[25] The new molecules **1**–**7**, being efficient in vivo anti-inflammatory, potent in vitro lipoxygenase inhibitory and antioxidant agents, with significantly reduced GI-toxicity are good candidates for potential use in neurodegenerative diseases.

Table 5. Gastrointestinal toxicity of indomethacin, naproxen, and the novel derivatives in rats

Compound	Dose (μmol/kg)	Mortality (%)[a]	Perforating ulcers (%)[b]	Body weight change[c]	Incidence of melena
Indomethacin	84	50	80	−14.9	+
1	84	0	0	+1.3	—
2	84	0	0	+3.9	—
Naproxen	1350	50	100	−15.8	+
3	1350	0	0	+3.5	—
4	1350	0	0	+1.0	—

[a] Dead per total × 100.
[b] Percent of animals developing perforating intestinal ulcers.
[c] In g/100 g body weight. Standard deviation of the weight change was always within 10% of the average value.

Increasing evidence indicates that several pathogenic mechanisms promoting atherosclerosis are also involved in neurodegenerative diseases. An insight into the factors determining the susceptibility to atherosclerosis as well as its long-term progression is of interest for the understanding of the evolution of such diseases as Alzheimer's.[26] It is known that hypercholesterolemia is a risk factor of SDAT.[27] Also cholesterol has been demonstrated to modulate the processing of amyloid precursor protein (APP) to amyloid β peptide $A\beta_{42}$.[28] In addition, apolipoprotein E_4 ($ApoE_4$) is a pathological finding in SDAT,[5,29] while the role of atherosclerosis in vascular dementia is well known.[30] It is reported that increased plasma cholesterol is related to neurodegeneration.[6] A number of known NSAIDs present a good anti-dyslipidemic action.[31] Furthermore, new potent and safer derivatives of classical NSAIDs, structurally related to the compounds reported herein, reduce considerably plasma LDL-cholesterol.[7] We have also demonstrated that antioxidant properties of novel anti-dyslipidemic compounds are beneficial for their action.[32] Thus, the novel agents of the present study were evaluated for their hypocholesterolemic activity and preliminary results are presented in Table 6. Compounds **2**, **4**, **5**, **6**, and **7** were administered ip to hypercholesterolemic rats and 24 h later the plasma total cholesterol (TC), LDL-cholesterol, and triglyceride (TG) concentrations were determined in blood taken from the aorta.[33] The results of this experiment demonstrate that the test compounds exhibit significant hypolipidemic activity.

In conclusion, the novel compounds, as designed, acquired all the desired properties, that is, in vivo and in vitro anti-inflammatory, antioxidant, low GI-toxicity, and considerable anti-dyslipidemic action. It seems that the in vivo actions are due to the whole molecular entity and not to any liberation of the parent drug in the organism. This is supported by their much lower GI toxicity compared with the parent NSAID, as well as by existing evidence that amide derivatives of indomethacin and naproxen are not hydrolyzed significantly in the body.[22,34] Furthermore, structurally related proline derivatives have been found to be powerful nootropic and antiamnesic agents. We believe that multicausal disorders, such as Alzheimer's disease, can be treated effectively with agents designed to act at different causes and stages of their pathogenesis. Thus, we expect that our approach will provide lead compounds that may be useful in slowing the progression or delaying the onset of this devastating disease. These molecules may also find useful applications in other pathologic conditions such as multiple sclerosis and atheromatosis, where inflammation and oxidative stress are also involved.[4]

Table 6. Effect of the test compounds on plasma total cholesterol, triglyceride, and LDL-cholesterol levels of tyloxapol-induced (200 mg/kg) hyperlipidemic rats

Compound	Dose (μmol/kg)	% decrease[a]		
		TC	LDL	TG
2	200	78.0**	48.4*	55.7*
4	56	58.0**	55.0*	68.7**
5	56	45.0*	53.5*	51.0
6	56	35.5	56.5*	25.0
7	56	40.0	52.0*	21.3

[a] Each value is the mean from 4 to 9 rats in two independent experiments. Statistical significance compared with hyperlipidemic controls.

* $P < 0.05$.

** $P < 0.001$ (Student's t test).

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