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Novel dual inhibitors of calpain and lipid peroxidation

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Abstract—A series of molecules with dual inhibitory activities on calpain and lipid peroxidation were synthesized. These hybrid compounds were built on the calpain pharmacophore 2-hydroxytetrahydrofuran linked to a set of antioxidants via a L-leucine linker. Compound **7**, the most potent in cellular calpain and lipid peroxidation inhibitions, provided effective protection against glial cell death induced by maitotoxin.

1. Introduction

Calpains, members of the thiol protease superfamily, are implicated in a number of patho-physiological processes. In particular, the role of calpain 1 in central nervous system conditions such as stroke, Parkinson's and Alzheimer's diseases, subarachnoid hemorrhage or head trauma, as well as in peripheral pathologies like muscular dystrophy, cataract, cardiac ischemia, restenosis or arthritis has been extensively studied.[1] Most of these pathologies are associated with inflammatory processes and the production of free radical species such as reactive oxygen species (ROS).

In pioneering experiments, we demonstrated that the combination of a calpain inhibitor (Z-LL-H)[2] and an antioxidant (BHT)[3] led synergistically to protection against glial cell death.[4] Consequently, we set up a chemical program aimed at designing molecules possessing both activities.

The free aldehyde group of Z-LL-H was regarded as a chemically reactive function incompatible with in vivo administration. Therefore the 2-hydroxy-tetrahydrofuran group, a masked aldehyde, was selected as a calpain pharmacophore[5a] suitable for our purposes. In this context, the biologically active species was hypothesized to be the hydroxyalkyl-aldehyde (the opened semiacetal),[5b] which is in equilibrium with the cyclic semiacetal. Thus, the available hydroxyalkyl-aldehyde forms a hemithioacetal with the cysteine residue of the calpain active site.

On the other hand, the selection of the antioxidant part of these hybrid compounds was facilitated by our previous work in the field of dual NOS-ROS inhibitors.[6]

Furthermore, we reasoned that these hybrid compounds should have balanced calpain inhibitory and free radical scavenging properties. Attention was therefore focused on a series of potent antioxidants (**1a**–**d**) as illustrated in Scheme 1.

As a first approach to the design of these dual inhibitors, the connection between the calpain pharmacophore and the antioxidant was inspired by the peptidic backbone of classical calpain inhibitors. Thus, the optimization of the molecules focused on the nature of the antioxidant and its impact on the calpain and lipid peroxidation inhibitory activities. A 4-step synthetic pathway (Scheme 1) was used to convert commercial (*S*)-leucine methyl ester·HCl, **2**, into compounds **6a**–**d**. Compound **2** was either condensed with trolox® **1a**–$\mathbf{CO_2H}$,

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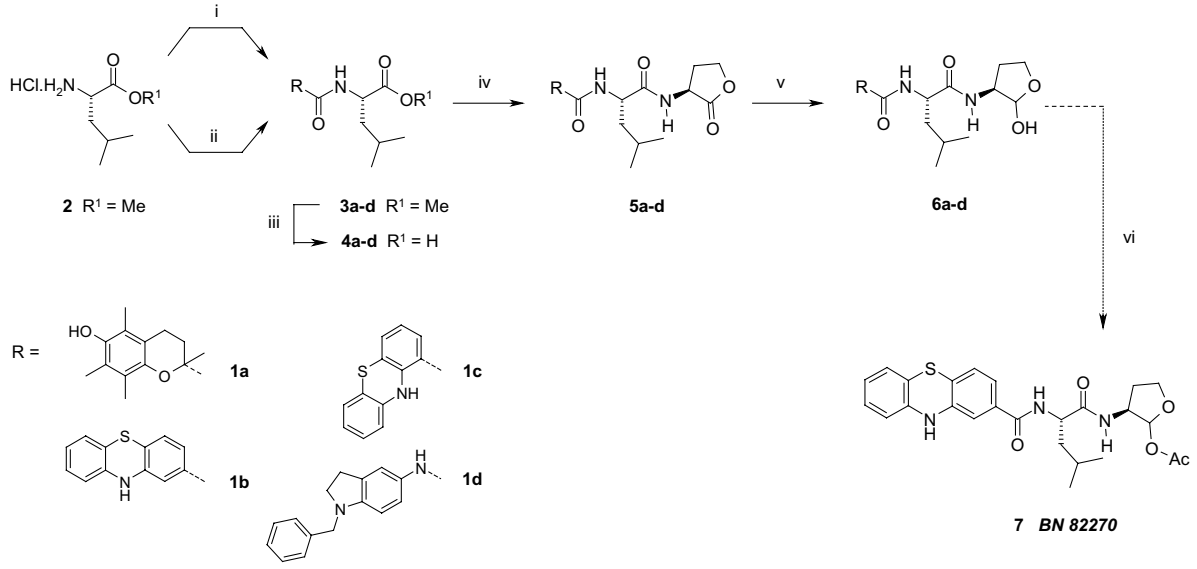


Scheme 1. Reagents and conditions: (i) **1a**–CO_2H, **1b**–CO_2H or **1c**–CO_2H, EDC, HOBT, Et_3N, DMF, 20 °C, 15 h; (ii) triphosgene, DIEA, **1d** (NH_2), 20 °C, 4 h, 70% ; (iii) LiOH, THF, H_2O, 20 °C, 2 h; (iv) (*S*)-α-amino-γ-butyrolactone·HBr, EDC, HOBT, Et_3N, DMF, 20 °C, 15 h; (v) DIBAL 3 equiv THF, −60 °C, 1 h; (vi) **6b**, Ac_2O, DMAP, 3 h, 20 °C, 70%.

phenothiazines **1b**–CO_2H[7] or **1c**–CO_2H[8] to afford carboxamides **3a**–**c**, or converted to the urea **3d** by reaction with **1d** (NH_2) in the presence of triphosgene and DIEA. The methyl esters of **3a**–**d** were saponified and the resulting carboxylic acids **4a**–**d** were condensed with commercial (*S*)-α-amino-γ-butyrolactone using EDC/HOBT to yield the lactones **5a**–**d** which, on reduction with DIBAL at −60 °C, afforded the hybrid molecules **6a**–**d**. It should be noted that each compound of the **6a**–**d** series exists as an equilibrated two-diastereomeric mixture. This is attributed to the reversible and nonstereoselective closing and opening of the semi-acetal ring, with intermediate formation of a transient hydroxyalkyl aldehyde, with traces of water.

The aminoindoline **1d** (NH_2)[9] was accessible (Scheme 2) through a short straightforward strategy from commercial 5-nitroindoline. Alkylation with benzyl bromide and reduction of **8** by a mixture of Raney Ni and hydrazine hydrate in EtOH readily gave **1d** (NH_2).

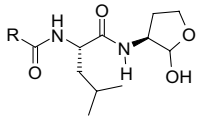
2. Results and discussion

The biological activities of compounds **6a**–**d** were evaluated in a human calpain 1 enzyme assay,[10] with Suc-Leu-Tyr-AMC as substrate, and the antioxidant potency assessed by their ability to inhibit Fe^{2+} induced lipid peroxidation (LPO) in rat brain microsomes.[11]

Z-LL-H and BHT (2,6-di-*tert*-butyl-4-methylphenol) were chosen as reference compounds for calpain and LPO test, respectively. The results are shown in Table 1.

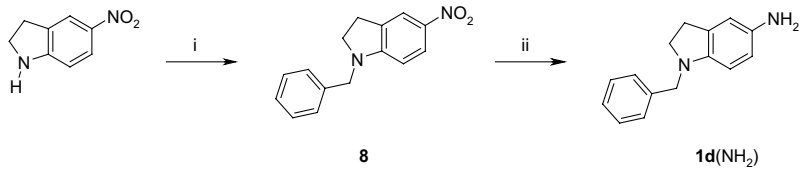
This study on dual calpain and LPO inhibition began with the synthesis of the Trolox® based compound **6a**, which confirmed the synthetic feasibility of these hybrid compounds. The promising calpain inhibitory activity ($IC_{50} = 147$ nM) of **6a** demonstrated that, despite the

Table 1. Calpain and lipid peroxidation inhibitions for **6a**–**d**



Compds	R	Calp Inh. IC_{50}, nM	LPO Inh. IC_{50}, nM
6a	**1a**	147	1700
6b	**1b**	22.5	70
6c	**1c**	82	3600
6d	**1d**	20	2340
Z-LL-H		4.9	na
BHT		na	3250

na, not active.



Scheme 2. Reagents and conditions: (i) Benzyl bromide, NaH, DMF, 15 h, 20 °C, 92%; (ii) N_2H_4, H_2O, Raney Ni, EtOH, reflux, 4 h, 78%.

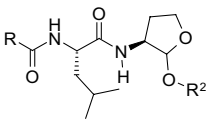
steric bulk of the antioxidant moiety, this compound was capable of binding to the calpain active site. Previous work in the domain of hybrid compounds, showed that Trolox® based NOS inhibitors,[6] exhibited strong lipid peroxidation inhibition, however, in **6a**, the Trolox® moiety conferred a weaker activity than expected. Thus, the antioxidant potency of a molecule not only depends on the nature of the antioxidant moiety used, but also on the molecular structure as a whole. In the phenothiazine series, the advantageous contribution of the relatively flat heterocyclic ring was highlighted by the performance of compounds **6b** and **6c** in the calpain test. The 2-subsituted phenothiazine **6b** had better affinity than the 1-substituted phenothiazine **6c** for the calpain active site. The superior inhibitory potency of **6b** in the calpain test ($IC_{50} = 22.5$ nM) was accompanied by a dramatic improvement in the free radical scavenging activity with an $IC_{50} = 70$ nM. A final attempt to modify the antioxidant moiety of the molecule was illustrated by the use of the benzyl-aminoindoline **1d**. The compound **6d** proved to be as active as **6b** in the calpain test, however with lower antioxidant activity. A structural comparison of **6b** and **6d** demonstrated that a nearly planar heterocyclic system was required at the N-terminal position to achieve a high level of calpain inhibition.

In the light of these results compounds **6b** and **6d**, were chosen for further in vitro studies involving cellular calpain inhibition[12] in C6 glial cells and protection against C6 glial cell death induced by maitotoxin.[13] The latter requires calpain inhibition associated with free radical scavenging activity to protect the cells. Z-LL-H and BHT were used as reference compounds (Table 2).

Surprisingly, **6d** was devoid of activity in the glial cell test. Whether **6d** was unable to penetrate into the cell or whether it was degraded inside the cell remains unclear. On the contrary, **6b** was active in the C6 calpain inhibition test, although less potent than Z-LL-H. The superiority of **6b** versus Z-LL-H was revealed in the protection against cell death test, where **6b** had an $IC_{50} = 38.5$ μM whereas Z-LL-H, and BHT were weakly active (100 μM). Since **6b** showed good enzyme inhibition (Table 1), we hypothesized that its modest activity in the C6 cell test was a consequence of moderate cell permeation.

Accordingly, the free hydroxyl group was masked in order to enhance the cellular activity. Compound **6b** was transformed into **7** with acetic anhydride in the presence of DMAP (Scheme 1). This acetylation of the semi-acetal ring generated a non-equilibrated two-diastereomeric mixture of **7** with a ratio of (83/17) as determined by NMR experiments. In the human calpain 1 assay, as expected, **7** did not show any significant activity ($IC_{50} > 1$ μM). However, to our satisfaction, **7** was 1.8-fold more active than **6b** in the C6 cell test and 2.5-fold in the necrosis test, which demonstrated that **7** was able to regenerate **6b** inside the cell. Furthermore, the LPO inhibitory activity was conserved for **7**.

Table 2. Calpain and cell death inhibitions in C6 glial cells for compounds **6b**, **6d** and **7**



Compds	R	R^2	C6 Calp Inh. IC_{50}, μM	Cell death Inh. IC_{50}, μM
6d	**1d**	H	>100	nt
6b	**1b**	H	23.49 ± 2.12	38.5 ± 5.77
7	**1b**	Ac	13.34 ± 1.23	15.5 ± 1.46
Z-LL-H			3.04 ± 0.33	16% at 100 μM
BHT			na	21% at 100 μM

nt, not tested; na, not active.

3. Conclusion

This study demonstrates the synthetic feasibility of obtaining dual calpain/LPO inhibitors in spite of the steric bulk of the antioxidant moiety. Calpain was indeed very sensitive to the nature of the antioxidant group and 2-substituted phenothiazines proved to be superior to the other antioxidants in the calpain and LPO tests. Compound **6b** was not only a potent inhibitor of isolated calpain but also a powerful free radical scavenger, with comparable activities in both tests. Compound **7**, a prodrug of **6b**, proved to be superior to **6b** in cellular assays and furthermore, these hybrid compounds were much more active than Z-LL-H in the protection against C6 glial cell death. Compound **7** is currently undergoing further in vivo evaluation.

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