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Novel dual inhibitors of calpain and lipid peroxidation with enhanced cellular activity

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Abstract—A series of dipeptides with dual inhibitory activities on calpain and lipid peroxidation were prepared to target the intracellular calpain. This optimization program focused on the variations of the linker and the N-terminal amino acid of the peptidic core. Two compounds **6d-05** and **6d-08** exhibited potent intracellular calpain inhibition. The polar surface area and the number of rotors appeared to be critical descriptors to account for the behavior of these hybrid molecules in the cellular calpain assay.

Calpains are a superfamily of Ca^{2+} dependent cysteine proteases implicated in the modulation of cellular function. Calpains are present in almost all human cells and have been implicated and extensively studied in numerous central or peripheral degenerative diseases.[1] Most of these pathophysiological situations are associated with inflammation and an enhanced formation of free radicals. Such high levels of free radicals have deleterious consequences for cells. In previous experiments, we demonstrated that the combination of an antioxidant and a calpain inhibitor protected glial cells from death in a synergistic manner.[2]

In the literature, extremely potent calpain inhibitors have been disclosed. Nevertheless, because of the intracellular calpain location, these inhibitors do not perform well in cellular inhibition assays.[3] Optimization criteria stemming from the SAR studies seldom match the prerequisites for intracellular activity. Thus, the design of powerful, cell penetrating, inhibitors remains a challenge.

Previously, we reported the discovery and synthesis of a series of potent dual inhibitors of lipid peroxidation and cellular calpain.[4] In continuation of this program on hybrid molecules, we herein describe recent achievements in this field. The antioxidant properties of our molecules originated from phenothiazine and the calpain pharmacophore from the 2-hydroxy-tetrahydrofuran group. This masked aldehyde is thought to form a hemithioketal with the cysteine residue at the active site of the enzyme and therefore acts via reversible calpain inhibition.[4]

In parallel, a theoretical calculation approach was set up aimed at the identification of molecular descriptors which best describe the cellular activity of these molecules.

This series of molecules was accessible through a straightforward four-step synthetic pathway (Scheme 1). The starting aminoacid derivative **2**[5] was prepared in three steps from commercially available Cbz-L-leucine and (*S*)-α-amino-γ-butyrolactone. The chain elongation of **2** with Cbz-protected aminoacids was performed with HBTU as the coupling reagent and was followed by hydrogenolysis of the N-protecting group to yield the primary amines **4**. Coupling with either the alkyl carboxylic acid derivatives of phenothiazine, **1a**-CO_2H,[6]

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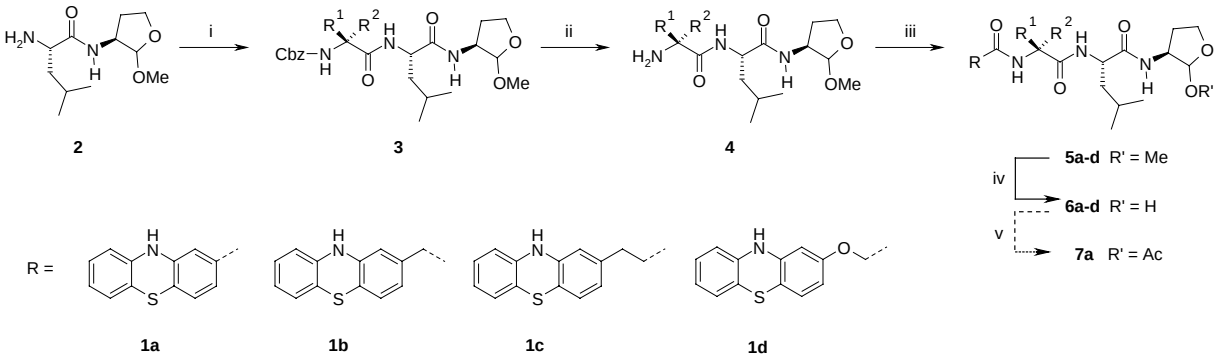


Scheme 1. Reagents and conditions: (i) CbzHN-C(R^1R^2)-CO_2H, HBTU, DIEA, DMF, 20 °C, 15 h; (ii) H_2 Pd/C 10%, CH_2Cl_2/EtOH, 20 °C, 2 h; (iii) **1a**-CO_2H, **1b**-CO_2H, **1c**-CO_2H, or **1d**-CO_2H, HBTU, DIEA, DMF, 20 °C, 15 h; (iv) 2 N HCl, acetone/H_2O, 20 °C, 15 h; (v) **6a**, Ac_2O, DMAP, 20 °C, 4 h, 60%.

1b-CO_2H,[7] and **1c**-CO_2H,[8] or with the ether analog **1d**-CO_2H[9] afforded a series of dipeptides **5a–d** with a different pattern of substitution between the peptidic core and the antioxidant. Deprotection of the ketal group was performed under acidic conditions to give access to the final hybrid compounds **6a–d**. It is noteworthy that each compound of the **6a–d** series exists as an equilibrated mixture of two diastereoisomers due to the reversible closing and opening of the hemiketal group in solution.[4] One of these compounds, **6a**, was acetylated with Ac_2O to generate a prodrug, **7a**.

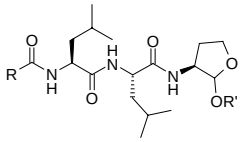
The biological activities of compounds **6a–d** and **7a** were evaluated in a human isolated calpain 1 enzyme assay,[10] with Suc-Leu-Tyr-AMC as substrate, and the intracellular calpain inhibition was performed in glioma C6 cells with the same substrate, in the presence of maitotoxin as a calpain activation inducer.[11] The antioxidant potency of the final molecules was assessed by their ability to inhibit Fe^{2+} induced lipid peroxidation (LPO) in rat brain microsomes.[12]

Z-LL-H[13] and BHT[14] (2,6-di-*tert*-butyl-4-methylphenol) were chosen as reference compounds for the calpain and LPO tests, respectively. The results are shown in Table 1.

A double L-leucine peptidic chain was selected as the scaffold for the evaluation of the phenothiazine connection to the rest of the molecule (Table 1). As expected, phenothiazines gave a series of very potent antioxidants which were at least as good as BHT in the LPO test. Whereas the shortest compound, **6a**, built on the 2-carboxyphenothiazine (**1a**) residue moderately inhibited the isolated human calpain (IC_{50} = 85.5 nM), it proved to be slightly more active than Z-LL-H in the cellular calpain assay (IC_{50} = 1.8 μM). To see whether a complementary prodrug strategy would be useful, compound **7a** was prepared. However, **7a** did not show any improvement compared to the parent drug **6a** (IC_{50} = 1.7 μM). Consequently, our efforts were mainly devoted to the optimization of the N-terminal part. Further increases of the alkyl chain (**1b** and **c**) in compounds **6b** and **c** were not favorable as shown by the drop in activity (**6c**, IC_{50} = 10 μM) in the cellular calpain test.

Conversely, the analogous compound **6d-08**, bearing an oxygen atom, outperformed not only Z-LL-H in the intracellular assay (IC_{50} = 0.6 μM) but also the other molecules of the series in the LPO test (IC_{50} = 333 nM). This beneficial double effect, consecutive to the exchange of residue **1c** by **d** in the molecule, appointed **6d-08** as the new lead compound.

Table 1. Human calpain 1, lipid peroxidation, and intracellular calpain inhibitions for **6a–d** and **7a**

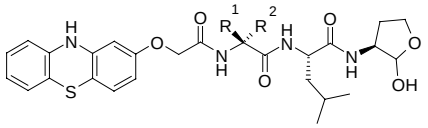


Compound	R	R′	Human calpain 1 IC_{50}[a] (nM)	LPO IC_{50}[a] (nM)	Intracellular calpain IC_{50}[a] (μM)
Z-LL-H	—	—	4.9	na	3
BHT	—	—	—	3250	—
6a	1a	H	85.5	1095	1.8
7a	1a	Ac	905	2310	1.7
6b	1b	H	55.4	966	2.5
6c	1c	H	150	3296	10
6d-08	1d	H	105	333	0.6

na = not active.

[a] Values are means of three experiments.

Table 2. Human calpain 1 and intracellular calpain inhibitions with molecular descriptors for **6d-01** to **6d-12**



Compound	R^1	R^2	Human calpain 1 IC_{50}[a] (nM)	Intracellular calpain IC_{50}[a] (μM)	pRatio[b]	Rotors[14]	FISA[c]
6d-01	—	—	8	63	−0.9	11	178.6
6d-02	Me	—	25	15	0.22	11	151.82
6d-03	Me	Me	138	30	0.66	11	125.38
6d-04	Et	—	88.6	0.8	2.05	12	138.13
6d-05	*n*-Pr	—	150.9	0.6	2.4	13	134.54
6d-06	*i*-Pr	—	75.9	1	1.88	12	123.83
6d-07	—	*i*-Pr	553.2	16	1.54	12	147.67
6d-08	*i*-Bu	—	105	0.6	2.24	13	133.82
6d-09	*t*-Bu	—	354.1	4	1.95	12	121.91
6d-10	Ph	—	222	5	1.65	12	137.04
6d-11	HO-CH_2	—	48.7	3	1.21	13	186.72
6d-12	H_3C-CH(OH)	—	38	2	1.28	13	167.53

[a] Values are means of three experiments.
[b] pRatio = −log(intracellular calpain/human calpain 1).
[c] FISA: polar surface area.[15]

To thoroughly study the SAR around **6d-08**, an additional synthetic program was set up to replace the L-leucine of the linker by diversely substituted α-aminoacids (Table 2). The significance of the lateral chain was apprehended by using glycine as a starting point in **6d-01**, this compound behaving as an excellent calpain inhibitor (IC_{50} = 8 nM). Attempts to improve this inhibition by a variation of the lateral chain in the linear or branched alkyl series led only to a decrease in inhibitory potency. Moreover, neither the *gem*-dimethyl substitution in **6d-03** nor the D-isomer of valine in **6d-07** were valuable. A phenyl residue used as a flat surrogate of the alkyl chain in compound **6d-10** (IC_{50} = 222 nM) did not perform better than **6d-01**. To glance over less lipophilicity, two alcohol derivatives **6d-11** and **6d-12** were prepared and evaluated. Although they displayed potent IC_{50}s against calpain (≈40 nM), they did not perform better than their alkyl counterparts. These results illustrated that calpain was very sensitive to the pattern of substitution and that glycine was the most suitable amino acid at this region.

Conversely, the peptidic backbone modifications of the **6d** series had a limited impact on free radical scavenging. All the compounds had IC_{50}s in the same range of activity (≈300–400 nM) in the LPO assay. The antioxidant potency of the molecules was more influenced by the phenothiazine substitution (Table 1) than by the rest of the structure.

To complete the inhibitory activity profile of compounds **6d-01** to **6d-12**, they were tested against cathepsin B, caspase 3, and α-chymotrypsin. Indeed, these molecules were potent inhibitors of cathepsin B, IC_{50}s were equivalent to those calculated for the calpain inhibition. In contrast, caspase 3 and α-chymotrypsin were not inhibited by the compounds.

On the other hand, this series of compounds was evaluated in a cellular calpain assay. Previously, we checked that Suc-Leu-Tyr-AMC was not a substrate for cathepsin B to ensure reliability of the results. Surprisingly, compound **6d-01** which behaved as an excellent calpain inhibitor displayed poor activity in the cellular assay (IC_{50} = 63 μM). This could be attributed either to a lack of permeation or to a degradative process occurring inside the cell. Among the methyl analogs, the L-alanine derivative **6d-02** was able to recover some activity, whereas the *gem*-dimethyl **6d-03** was not. As this gem di-substitution was likely to prevent this compound from proteolysis, the only remaining hypothesis is based on the permeation properties of the molecules. Increasing the size of the lateral chain from ethyl to isobutyl, **6d-04** to **6d-08**, led to a series of powerful inhibitors (IC_{50} = 0.6 μM for **6d-05** and **6d-08**) of cellular calpain, except for **6d-07**. The L-isomer **6d-06** was sixteen times more potent than the D-isomer **6d-07** in the cellular calpain inhibition assay which underlines the usefulness of the L-isomer for this chemical series. Even if these compounds were, on average, 20 times less active than Z-LL-H on the isolated enzyme, they displayed a consistent four to five times better performance in the cellular assay. In the same way, the most bulky and lipophilic compounds of this series, **6d-09** and **6d-10**, although considerably less active than Z-LL-H on calpain, exhibited similar performance against cellular calpain. Interestingly, the two alcohol derivatives **6d-11** and **6d-12** also performed as well as Z-LL-H.

The IC_{50}s of the intracellular calpain inhibition (Table 2) correspond to a 4 h incubation time of the molecules including 3 h in the presence of maitotoxin. It is noteworthy that this inhibition remained constant between 90 min and 3 h showing that our molecules gave a prolonged inhibition.

In the light of these results, the cellular activity could not be related to either stability or lipophilicity (C log *P*).[3d] A theoretical calculation approach was therefore set up to get an insight into the parameters

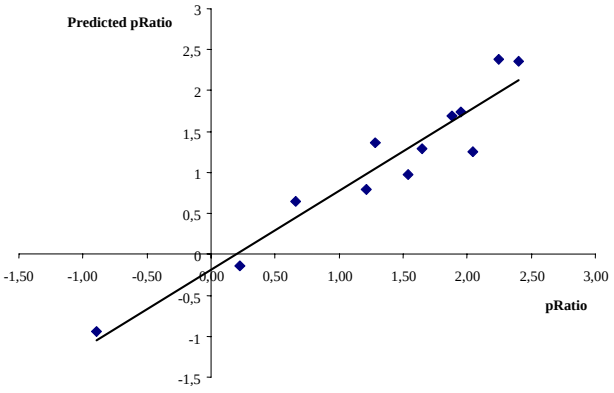


Figure 1. Predicted pRatio versus experimental pRatio of compounds **6d-01** to **6d-12**.

which describe the behavior of these molecules in the cellular assay. For this purpose 3D coordinates of molecular structures were generated by Corina 3.2 (Molecular Networks GmbH) using the 'write hydrogen atoms' and the 'neutralize formal charges' options. The resulting 3D structures were then used to generate molecular descriptors such as rotors[15] and FISA[16] using QikProp 2.2 (Schrodinger) (Table 2). Multiple linear regression analysis highlighted the significance of the number of rotors and the polar surface area (FISA) of the molecules. The predicted pRatio could thus be described as a combination of these two descriptors:

$$\textit{predicted}\ \text{pRatio} = -6.479 + (0.987) * \text{rotor} - (0.028) * \text{FISA}.$$

The excellent correlation and predictability of this model, $q^2 = 0.846$ (cross-validated r^2), between the predicted pRatio and the experimental pRatio (Table 2) are illustrated in Figure 1.

Our study has led to the design of a series of dipeptides which are potent calpain and LPO inhibitors. Some of them, **6d-05** and **6d-08**, show strong inhibitory potencies in cellular assays with IC_{50}s below 1μM and with a limited (4- to 6-fold) decrease in cellular activity compared to enzymatic inhibition. With the help of a calculation approach, we were able to demonstrate that, in this series of compounds, the polar surface area and the number of rotors of a molecule adequately described their performance in this cellular calpain inhibition assay.

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15. Number of nontrivial (not CX3), nonhindered (not alkene, amides, and small ring) rotatable bonds in the molecule.
16. Hydrophilic component of the solvent accessible surface area.