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Communications to the Editor

D-Amino Acid Containing, High-Affinity Inhibitors of Recombinant Human Calpain I†

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Calpains are calcium-activated neutral proteases belonging to the papain superfamily of cysteine proteases. Two major forms of calpains have been identified: calpain I (μ-calpain) and calpain II (m-calpain), which require low and high micromolar Ca^{2+} concentrations for activation, respectively. While calpain II is the predominant form in many tissues, calpain I is thought to be the predominant form activated during pathological conditions in nervous tissues.[1] Calpain I has been implicated in many nervous system disorders including stroke, Alzheimer's disease, motor neuron damage, and muscular dystrophy; thus, in recent years, calpain inhibition has become an important pharmacological goal.[2] Our involvement in inhibiting calpain emerged from our interest in finding new therapeutics[3] to treat stroke, one of the leading causes of mortality in the western hemisphere. An episode of stroke (focal cerebral ischemia) initiates a chain of biochemical events resulting in a delayed neurodegeneration. Calpains become activated by the increased intraneuronal calcium concentrations associated with ischemia. Activated calpains, in turn, degrade neuronal structural proteins, contributing to the neurodegenerative process.

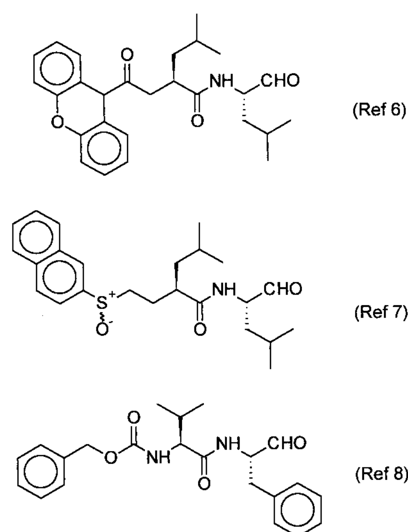

Figure 1.

Potent peptide-based reversible (aldehyde and α-ketocarbonyl)[4] and irreversible (halomethyl ketone, diazomethyl ketone, epoxysuccinate, and acyloxymethyl ketone)[5] inhibitors of calpain have been reported. In all of these inhibitors, calpain tolerated a range of amino acids at P_1. *However, in potent inhibitors, the P_2 amino acid was always either L-Leu or L-Val, suggesting this could be a strict structural requirement of calpain at the P_2 site.*[4,5] Recently, we disclosed potent nonpeptidic ketomethylene and carbamethylene (P_2-P_3) containing calpain I inhibitors derived from xanthene[6] and thionaphthalene,[7] respectively (Figure 1). Our work revealed that the NH at the P_2 site of a potent dipeptide inhibitor can effectively be replaced by a CH_2, provided an aromatic moiety is employed in the P_3 region. We reasoned that the requirement of calpain I for an isobutyl group (from leucine) or an isopropyl group (from valine) might be steric in nature; either of these moieties could occupy the same pocket of the enzyme's S_2 subsite. In designing our target molecule, we decided to replace the P_2-isopropyl group in the known potent calpain I

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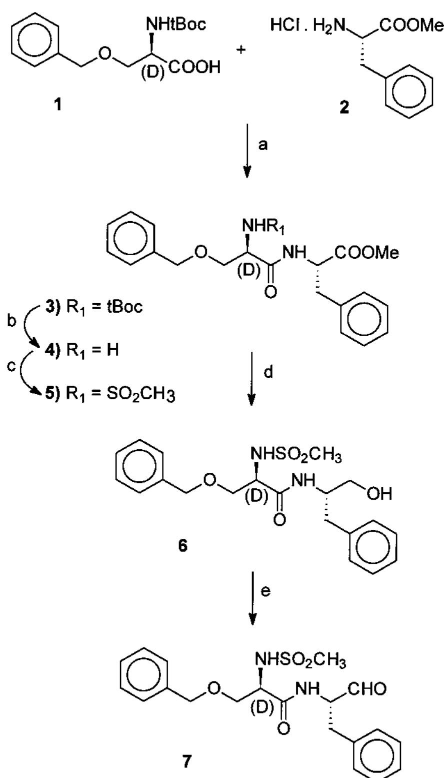
Scheme 1[a]



[a] Reagents: (a) BOP, 1-HOBt, NMM, DMF, 0 °C to room temperature, 2 h; (b) 90% TFA, CH_2Cl_2, room temperature, 3 h; (c) CH_3SO_2Cl, NMM, CH_2Cl_2, 0 °C to room temperature, 1 h; (d) $LiBH_4$, THF, 0 °C to room temperature, 1 h or $NaBH_4$, MeOH, 0 °C to room temperature, overnight; (e) Dess−Martin periodinane, CH_2Cl_2, 0 °C to room temperature, 1 h.

inhibitor, Cbz-Val-Phe-H (MDL 28170, Figure 1),[8] by a sulfonamido group. At the same time, as described before, we wished to incorporate an aromatic moiety in the P_3 region attached by a spacer to the P_2 site.

Chemistry. Scheme 1 depicts a representative synthesis of the target compound **7**.[9] Commercially available Boc-D-Ser(Bn) (**1**) was coupled with L-Phe-OMe hydrochloride salt (**2**) to generate the dipeptide **3**; the *t*-BocNH group in **3** was converted to amine **4** which was coupled with methanesulfonyl chloride to generate the sulfonamide **5**. Reduction of the ester group in **5** produced the dipeptide alcohol **6** which underwent Dess−Martin oxidation to generate the target aldehyde **7**. Alternatively, compound **6** can be prepared by coupling methanesulfonyl-D-Ser(Bn) and (*S*)-phenylalaninol; methanesulfonyl-D-Ser(Bn) can be generated from D-Ser(Bn) by treatment with methanesulfonyl chloride in the presence of aqueous NaOH.

In Vitro Biology and Discussion. The biological activities of the compounds were determined using recombinant human calpain I (rh calpain I), prepared as described by Meyer et al.,[10] with Suc-Leu-Tyr-MNA (Enzyme Systems Products, Dublin, CA) as the substrate.[6] Table 1 displays the inhibitory data for a series of P_2-D-Ser(Bn) derived inhibitors and the reference compound Cbz-Val-Phe-H. All reaction progress curves in the presence of inhibitor were linear, consistent with rapid equilibrium binding of the compounds to calpain. As shown, P_1-Phe is preferred over Abu and Leu (cf. **7** vs **8** and **9**). Interestingly, incorporation of P_1-Lys(SO_2-Ph) and Tyr(Bn) generated **10** and **11** (K_i values of 2 and 4 nM, respectively), the two most potent compounds of the series; thus, it appears that the S_1 pocket of the enzyme tolerates large hydrophobic groups from the P_1 position. It should be noted that the reference compound, Cbz-Val-Phe-H, shows a K_i value of 8 nM in this assay. Variation in sulfonamide groups generated compounds **7**, **12**, **13**, and **14**. While methane- and ethanesulfonamides were preferred over benzenesulfonamide (cf. **7** and **12** vs **13**), 2-thienylsulfonamide (**14**) was equipotent, revealing the beneficial effect of an additional heteroatom in this region. Compound **15**, which contains an *N*-methyl methanesulfonamide, is approximately 46 times less potent than compound **7**, suggesting that the NH of the sulfonamide moiety of compound **7** is involved in energetically beneficial binding. The aldehyde enzyme reactive group is approximately 16 times preferred over the α-ketocarboxamide group (cf. **7** vs **16**). Compound **16** (K_i 130 nM) is, however, comparable to a reference L,L-dipeptide α-ketocarboxamide, Cbz-Leu-Abu-CONHEt[4c] (K_i 170 nM, in this assay).

Table 1. P_2-D-Ser(Bn)-Derived Inhibitors of Recombinant Human Calpain I[a]

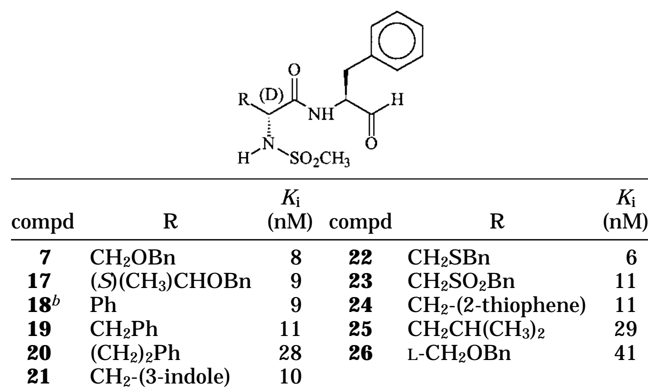
compd	X	R_1	R_2	R_3	K_i (nM)
7	H	Bn	H	CH_3	8
8	H	Et	H	CH_3	36
9	H	*i*Bu	H	CH_3	66
10	H	$(CH_2)_4NHSO_2Ph$	H	CH_3	2
11	H	$CH_2C_6H_4$-p-OBn	H	CH_3	4
12	H	Bn	H	Et	9
13	H	Bn	H	Ph	22
14	H	Bn	H	2-thienyl	9
15	H	Bn	CH_3	CH_3	370
16	CONHEt	Bn	H	CH_3	130
Cbz-Val-Phe-H					8

[a] $n \geq 3$ in all cases. Assay conditions as described previously.[5a] The K_m for Suc-Leu-Tyr-MNA = 0.5 mM. Values for IC_{50} were determined and converted to K_is using the expression $K_i = IC_{50}/(1 + S/K_m)$, assuming a competitive mechanism of inhibition.[12] Replicate determinations of K_i agree within 25%.

Table 2 displays the inhibitory activity of a series of compounds with varied P_2-D-amino acids (except **26**, see below). D-Ser(Bn), D-Thr(Bn), D-Phgly, D-Phe, D-Trp, and D-(thiophen-2-yl)Ala (**7**, **17**, **18**, **19**, **21**, **24**) are all well tolerated at P_2. However, D-Phe at P_2 (**19**) is approximately 2.5 times more potent than D-Homophe at P_2 (**20**). D-Cys(Bn) (**22**) and the corresponding sulfone analogue (**23**) at P_2 also maintain potency. Interestingly, incorporation of D-Leu at P_2 results in a ca. 4 times less potent compound (cf. **25** vs **7**), supporting our original hypothesis that the presence of an aromatic moiety in the P_3 region is beneficial. Finally, incorporation of L-Ser(Bn) at P_2 results in a greater than 5-fold decrease in potency (cf. **26** vs **7**) revealing the importance of the D configuration at P_2 in this series of compounds.

Evaluation of the Site of Binding. It is very difficult to demonstrate competitive inhibition kinetics with calpain by the classic method of examining the effect of compounds at various concentrations of substrate. Calpain substrates cannot be varied across a

Table 2. Variation of P_2-D-Amino Acids: Potency against Recombinant Human Calpain I[a]



compd	R	K_i (nM)	compd	R	K_i (nM)
7	CH_2OBn	8	**22**	CH_2SBn	6
17	(*S*)(CH_3)CHOBn	9	**23**	CH_2SO_2Bn	11
18[b]	Ph	9	**24**	CH_2-(2-thiophene)	11
19	CH_2Ph	11	**25**	$CH_2CH(CH_3)_2$	29
20	$(CH_2)_2Ph$	28	**26**	L-CH_2OBn	41
21	CH_2-(3-indole)	10			

[a] $n \geq 3$ in all cases. Values for IC_{50} were determined and converted to K_is using the expression $K_i = IC_{50}/(1 + S/K_m)$, assuming a competitive mechanism of inhibition.[12] Replicate determinations of K_i agree within 25%. [b]Elemental analysis: $C_{18}H_{20}N_2O_4S{\cdot}0.75H_2O$; calc H, 5.79; found H, 5.30.

sufficiently large concentration range to permit determination of the mechanism of inhibition, due either to limited solubility of the substrate or to substrate inhibition. To determine whether this novel series of compounds binds at the active site of calpain I, protection against inactivation experiments were performed. In this experiment, calpain I is preincubated with the test compound, followed by the addition of E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane), an irreversible inhibitor of calpain. By analogy with other well-studied cysteine proteases, E-64 is presumed to covalently label the enzyme's active site cysteine. The enzyme is then diluted into an assay mixture and its residual activity determined. The dilution factor is sufficiently large that any bound test compound will dissociate from the enzyme and the remainder will be at a concentration well below its IC_{50}. If the test compound and E-64 bind in a mutually exclusive manner, no loss of activity should be observed, while inactivation by E-64 should be apparent if the two compounds occupy distinct sites. Experiments with methanesulfonyl-D-Leu-Leu-H (**27**, K_i 68 nM), a less potent analogue, reveal (data not shown) that this compound protects against inactivation of calpain I by E-64, consistent with the binding of both compounds to a common site on the enzyme. However, this result should not be interpreted to demonstrate that both compounds bind in exactly the same way. For example, it is possible that compound **27** could react with the active site cysteine, but might lie in the pocket on the P′ side of the active site. Such a binding motif would still result in protection against inactivation by E-64, even though the two binding sites would be at least partially distinct. Elucidation of the exact nature of binding of this class of inhibitors to the enzyme thus requires determination of an X-ray crystal structure of the enzyme–inhibitor complex.

Specificity data. The selectivity of a subset of inhibitors was tested against several representative proteases. Table 3 lists the inhibitory data for compounds **7**, **10**–**12**, **17**, **19**, **22**, and **24** and the reference compound, Cbz-Val-Phe-H. The closely related enzyme calpain II is inhibited by all compounds tested with K_i values which are indistinguishable (except compound **10**) from those obtained against calpain I. Cathepsin B is a related cysteine protease which is sensitive to inhibition by L,L-dipeptide aldehydes. While both the reference compound and the parent compound **7** show a similar preference (ca. 3–4-fold) for calpain I over cathepsin B, compounds **11** and **19** both prefer calpain I by 11-fold over cathepsin B. Table 3 also displays the inhibitory activity of these compounds against α-chymotrypsin, a serine protease which is inhibited by other peptidyl P_1-phenylalaninals. Excellent selectivity was observed with respect to inhibition of chymotrypsin. A second serine protease, thrombin, which displays a distinct P_1 specificity, also was not inhibited (Table 3).

Table 3. Specificity Data for Compounds **7**, **10**–**12**, **17**, **19**, **22**, and **24**[a]

	K_i (nM)			% inh at 10 μM	
compd	calpain I	calpain II[b]	cathepsin B[c]	thrombin[d]	α-chymotrypsin[e]
7	8	5	32	0	23
10	2	17	4	0	13
11	4	5	45	0	0
12	9	9	45	9	15
17	9	7	44	0	3
19	11	13	120	0	12
22	6	7	41	7	16
24	11	11	23	0	0
Cbz-Val-Phe-H	8	*f*	24	*f*	*f*

[a] $n \geq 3$ in all cases. Values for IC_{50} were determined and converted to K_is using the expression $K_i = IC_{50}/(1 + S/K_m)$, assuming a competitive mechanism of inhibition.[12] Replicate determinations of K_i agree within 25%. [b]Porcine kidney calpain II (Calbiochem) assayed under the same conditions employed for calpain I (K_m for Suc-Leu-Tyr-MNA = 0.4 mM). [c] Human liver cathepsin B (Athens Research and Technology), 50 mM NaOAc (pH 6.0), 1 mM EDTA, 1 mM DTT, 100 μM Z-Phe-Arg-AMC (K_m = 300 μM), 5% DMSO. [d]Human plasma thrombin (Sigma), 50 mM Tris-Cl (pH 7.5), 10 mM $CaCl_2$, 100 μM Z-Phe-Val-Arg-AMC (K_m = 60 μM), 5% DMSO. [e]Bovine pancreas α-chymotrypsin (Sigma), 50 mM HEPES (pH 7.5), 0.5 M NaCl, 100 μM Suc-Ala-Ala-Pro-Phe-AMC (K_m = 170 μM), 5% DMSO. [f]Not determined.

Table 4. Inhibitory Data for Compounds **7**, **10**–**12**, **17**, **19**, **22**, and **24** in Intact Cell Assay[a]

compd	IC_{50} (μM)	compd	IC_{50} (μM)
7	0.7	**19**	0.6
10	0.4	**22**	0.8
11	0.7	**24**	0.3
12	0.4	Cbz-Val-Phe-H	0.3
17	0.5		

[a] $n \geq 2$ in all cases.

Cellular Activity. To probe the ability of these compounds to penetrate cells and inhibit intracellular calpain I, we tested a set of compounds in an intact cell assay system. Treatment of Molt 4 cells (human leukemic T cell line) with calcium ion and an ionophore results in the elevation of intracellular calcium which, in turn, activates calpain I.[11] This is followed by calpain I-mediated cleavage of cytoskeletal proteins, including spectrin. Inhibition of formation of spectrin breakdown products (SBDPs) inside the cell by a compound measures its efficacy. Table 4 lists the inhibitory activities of the compounds **7**, **10**–**12**, **17**, **19**, **22**, and **24**, which showed good activity in the assay for calpain I inhibition. In general, these compounds also display good activity in this assay with IC_{50} values in the range of 0.3–0.8 μM. Thus, the compounds are cell-permeable and inhibit intracellular calpains.

Conclusion. In this paper, we described a series of novel and potent calpain inhibitors incorporating *N*-alkyl- or -arylsulfonyl-D-amino acids at P_2. To our knowledge, this is unprecedented in the design of substrate-based potent cysteine protease inhibitor. The compounds are cell-permeable and inhibit intracellular calpain I in a human cell line. This study reveals for the first time that, contrary to literature evidence, the presence of L-Leu or L-Val residue at P_2 is not a preferred structural requirement for a potent calpain I inhibitor; an *N*-alkyl- or -arylsulfonyl-D-amino acid at P_2 can bind with high affinity.

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References

(1) (a) Croall, D. L.; DeMartino, G. N. Calcium-Activated Neutral Protease (Calpain) System: Structure, Function and Regulation. *Physiol. Rev.* **1991**, *71*, 813−847. (b) One of the reviewers commented “Based on the paper by Brorson et al. (*Stroke* **1995**, *26*, 1259) m-calpain can also produce spectrin breakdown in hippocampal neurons. Therefore we cannot rule out m-calpain as having no contribution to neuronal damage under the same pathological conditions where μ-calpain is activated.” We thank the reviewer for bringing this fact to our attention.

(2) (a) Bartus R. T. The Calpain Hypothesis of Neurodegeneration: Evidence for a Common Cytotoxic Pathway. *The Neuroscientist* **1997**, *3*, 314−327. (b) Wang, K. K. W.; Yuen, P. Calpain Inhibition: an Overview of its Therapeutic Potential. *Trends Pharm. Sci.* **1994**, *15*, 412−419.

(3) Barinaga, M. Finding New Drugs to Treat Stroke. *Science* **1996**, *272*, 664−666.

(4) (a) Iqbal, M.; Messina, P. A.; Freed, B.; Das, M.; Chatterjee, S.; Tripathy, R.; Tao, M.; Josef, K. A.; Dembofsky, B.; Dunn, D.; Griffith, E.; Siman, R.; Senadhi, S. E.; Biazzo, W.; Bozyczko-Coyne, D.; Meyer, S. L.; Ator, M. A.; Bihovsky, R. Subsite Requirements for Peptide Aldehyde Inhibitors of Human Calpain I. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 539−544. (b) Mehdi, S. Cell-Penetrating Inhibitors of Calpain. *Trends Biol. Sci.* **1991**, *16*, 150−153. (c) Li, Z.; Ortega-Vilain, A.-C.; Patil, G. S.; Chu, D.-L.; Foreman, J. E.; Eveleth, D. D.; Powers, J. C. Novel Peptidyl α-Keto Amide Inhibitors of Calpains and Other Cysteine Proteases. *J. Med. Chem.* **1996**, *39*, 4089−4098. (d) Harbeson, S. L.; Abelleira, S. M.; Akiyama, A.; Barrett, R., III,; Carroll, R. M.; Straub, J. A.; Tkacz, J. N.; Wu, C.; Musso, G. F. Stereospecific Synthesis of Peptidyl α-Keto Amides as Inhibitors of Calpain. *J. Med. Chem.* **1994**, *37*, 2918−2929. (e) Li, Z.; Patil, G. S.; Golubski, Z. E.; Hori, H.; Tehrani, K.; Foreman, J. E.; Eveleth, D. D.; Bartus, R. T.; Powers, J. C. Peptide α-Keto Ester, α-Keto Amide, and α-Keto Acid Inhibitors of Calpains and Other Cysteine Proteases. *J. Med. Chem.* **1993**, *36*, 3472−3480.

(5) (a) Chatterjee, S.; Ator, M. A.; Bozyczko-Coyne, D.; Josef, K.; Wells, G.; Tripathy, R.; Iqbal, M.; Bihovsky, R.; Senadhi, S. E.; Mallya, S.; O'Kane, T. M.; McKenna, B. A.; Siman, R.; Mallamo, J. P. Synthesis and Biological Activity of a Series of Potent Fluoromethyl Ketone Inhibitors of Recombinant Human Calpain I. *J. Med. Chem.* **1997**, *40*, 3820−3828. (b) Angliker, H.; Anagli, J.; Shaw, E. Inactivation of Calpain by Peptidyl Fluoromethyl Ketones. *J. Med. Chem.* **1992**, *35*, 216−220. (c) Crawford, C.; Mason, R. W.; Wickstrom, P.; Shaw, E. The Design of Peptidyldiazomethane Inhibitors to Distinguish between the Cysteine Proteinases Calpain II, Cathepsin L, and Cathepsin B. *Biochem. J.* **1988**, *253*, 751−758. (d) McGowan, E. B.; Becker, E.; Detwiler, T. C. Inhibition of Calpain in Intact Platelets by the Thiol Protease Inhibitor E−64d. *Biochem. Biophys. Res. Commun.* **1989**, *158*, 432−435. (e) Huang, Z.; McGowan, E. B.; Detwiler, T. C. Ester and Amide Derivatives of E64c as Inhibitors of Platelet Calpains. *J. Med. Chem.* **1992**, *35*, 2048−2054. (f) Harris, A. L.; Gregory, J. S.; Maycock, A. L.; Graybill, T. L.; Osifo, I. K.; Schmidt, S. L.; Dolle, R. E. Characterization of a Continuous Fluorogenic Assay for Calpain I. Kinetic Evaluation of Peptide Aldehydes, Halomethyl Ketones and (Acyloxy)Methyl Ketones as Inhibitors of the Enzyme. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 393−398. (g) For subsite nomenclature for peptide-based inhibitors, see: Schechter, I.; Berger, A. On the Size of the Active Site in Protease. 1. Papain. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157−162.

(6) Chatterjee, S.; Iqbal, M.; Kauer, J. C.; Mallamo, J. P.; Senadhi, S.; Mallya, M.; Bozyczko-Coyne, D.; Siman, R. Xanthene Derived Potent Nonpeptidic Inhibitors of Recombinant Human Calpain I. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1619−1622.

(7) Chatterjee, S.; Senadhi, S.; Bozyczko-Coyne, D.; Siman, R.; Mallamo, J. P. Nonpeptidic Inhibitors of Recombinant Human Calpain I. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 287−290.

(8) Mehdi, S.; Angelastro, M. R.; Wiseman, J. S.; Bey, P. Inhibition of the Proteolysis of Rat Erythrocyte Membrane Proteins by a Synthetic Inhibitor of Calpain. *Biochem. Biophys. Res. Commun.* **1988**, *157*, 1117−1123.

(9) Details will be published in a full account of this work.

(10) Meyer, S. L.; Bozyczko-Coyne, D.; Mallya, S. K.; Spais, C. M.; Bihovsky, R.; Kawooya, J. K.; Lang, D. M.; Scott, R. W.; Siman, R. Biologically-Active Monomeric and Heterodimeric Recombinant Human Calpain I Produced Using the Baculovirus Expression System. *Biochem. J.* **1996**, *314*, 511−519.

(11) For experimental protocol, see ref 5a.

(12) Segel, I. H. *Enzyme Kinetics*; Wiley: New York, 1975; p 106.

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