Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 5, pp. 539-544, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00063-2

SUBSITE REQUIREMENTS FOR PEPTIDE ALDEHYDE INHIBITORS OF HUMAN CALPAIN I

Mohamed Iqbal, Patricia A. Messina, Bethany Freed, Manoj Das, Sankar Chatterjee, Rabindranath Tripathy, Ming Tao, Kurt A. Josef, Bruce Dembofsky, Derek Dunn, Eric Griffith, Robert Siman, Shobha E. Senadhi, William Biazzo, Donna Bozyczko-Coyne, Sheryl L. Meyer, Mark A. Ator, and Ron Bihovsky*

Cephalon, Inc., 145 Brandywine Parkway, West Chester, PA 19380-4245

Abstract: Dipeptide and tripeptide aldehydes have been evaluated as inhibitors of human calpain I. Dipeptide aldehydes are generally equipotent with tripeptide aldehydes. Calpain I possesses a rather stringent requirement for Leu at P_2, but accepts a variety of capping groups and amino acids at P_1 and P_3. Several new peptide aldehydes that are more potent than previously reported calpain I inhibitors have been identified.

Calpain I, a thiol proteinase widely distributed in mammalian cells, hydrolyzes various physiologically important proteins when activated by elevated intracellular concentrations of calcium ion. Inhibitors of calpain I may be useful for treating neurodegenerative disorders and other diseases.[1] Substrates positioned such that Leu occupies the S_2 subsite are efficiently cleaved by calpain.[2-4] In accord with this substrate preference, peptide aldehydes containing P_2 Leu are potent active-site calpain I inhibitors.[3-6] Several previous publications present potencies of peptide aldehyde calpain I inhibitors measured with calpains purified from different species, under differing conditions, with different substrates.[3-5] The reported IC_{50} values are inconsistent, even with respect to rank order of potency for standard inhibitors. Therefore, a comprehensive profile of peptide aldehyde SAR is lacking. In order to further define the structural requirements of calpain I inhibitors, we have evaluated a series of dipeptide and tripeptide aldehydes in which the capping group, P_1, P_2, and P_3 residues were systematically varied.

Experimental Section

Peptide aldehydes were prepared by previously described methods:[7] (1) oxidation of peptide alcohols with pyridine-SO_3 DMSO[5] or Dess-Martin reagent in CH_2Cl_2,[8] (2) reduction of *N*-methoxy-*N*-methyl amides with lithium aluminum hydride,[9] or (3) hydrolysis of peptide acetals with TsOH in acetone-water or HCl in THF-water.[7] The peptide aldehydes were purified by recrystallization or reverse-phase HLPC, and characterized by NMR, HPLC, and mass spectroscopy. Unless otherwise noted, L-amino acids are represented. Peptide aldehydes were evaluated as inhibitors of recombinant human calpain I at 20 °C, utilizing Suc-Leu-Tyr-MNA (0.2 mM; K_m = 0.4 mM) as substrate, as previously described.[10] Z-Leu-Nle-H (Calpeptin) was purchased from Novabiochem; Ac-Leu-Leu-Nle-H (Calpain Inhibitor I) and Ac-Leu-Leu-Met-H (Calpain Inhibitor II) were purchased from Boehringer Mannheim; Ac-Leu-Leu-Arg-H (Leupeptin) was purchased from Sigma. The

IC_{50} values (see Table 1) of these peptide aldehyde standards, as well as Z-Leu-Phe-H, Z-Leu-Met-H and Z-Val-Phe-H (MDL-28170, IC_{50} = 11 nM), are fairly consistent with those previously determined with human or rat erythrocyte calpain I, but are generally lower than those determined with porcine erythrocyte calpain I. [3-5] Although peptide aldehydes exist partially as hydrates in aqueous solution, the IC_{50} values in Table 1 are not corrected for the fraction of aldehyde which is hydrated.

Results

Table 1 summarizes the potencies of the peptide aldehydes evaluated in this investigation. A series of dipeptide aldehydes was prepared in which the P_2 substituent was varied, while P_1 was maintained as Leu, and the capping group was held constant as benzyloxycarbonyl (Z). In accord with literature precedent, Leu at P_2 affords a potent calpain I inhibitor (8 nM). *t*-ButylGly gives the most potent inhibitor of the series (4 nM). Incorporation of Val, Nle, and Ile at P_2 decreases potency 4- to 6-fold, with respect to Leu. *t*-ButylAla at P_2 (140 nM) decreases potency 20 times relative to Leu, though it differs only by an additional methyl group. Cha affords a weak inhibitor (580 nM) because it exceeds the spatial limitations of the S_2 subsite. Incorporation of amino acids at P_2 which are capable of hydrogen bonding does not increase potency. Thus, P_2 Gln (370 nM) and Ser (530 nM) afford weak inhibitors. Thr at P_2 gives a moderate inhibitor (190 nM), despite being essentially isosteric with Val. Aromatic amino acids are not beneficial at P_2: Phe and Trp afford moderate inhibitors (230 and 120 nM, respectively), and Tyr gives a poor inhibitor (>1,000 nM). *N*-Methylation of the P_2 residue essentially destroys inhibition, suggesting that the P_2 amide may form a hydrogen bond to calpain I. β-Ala at P_2 is devoid of any calpain I inhibitory activity, since the requisite side chain is lacking, and the amide bond is displaced. Overall, these data confirm that calpain I possesses a rather stringent requirement for the isobutyl side chain of Leu at P_2 in dipeptide aldehyde inhibitors.

A series of dipeptide aldehyde inhibitors was prepared in which the P_1 substituent was varied, while P_2 was Leu, and the capping group was benzyloxycarbonyl. In this series, Val (4 nM) and Nle (5 nM) at P_1 afford the most potent inhibitors, followed by *O*-benzylTyr (7 nM) and Leu (8 nM). Abu, Phe, Cha, and His at P_1 provide somewhat lower potency (10-15 nM). Thus, the S_1 subsite of calpain I accommodates a range of aromatic and branched or unbranched aliphatic side chains. The methyl side chain of Ala and the hydrogen of Gly are insufficient for optimal recognition (120 and 300 nM, respectively). Although the side chain of Met is only slightly larger than that of Nle, Met at P_1 decreases potency 6 times relative to Nle. The tertiary-butyl side chain of *t*-BuGly is also nonoptimal (35 nM). *N*-Methylation of the P_1 Leu residue attenuates potency several hundred times, suggesting that the P_1 amide participates in hydrogen bonding to calpain I. P_1 β-Ala (>>10,000 nM) and β-benzyl-β-Ala (>1,000 nM) provide very poor inhibitors because the P_1 amide bonds and P_2 residues are displaced within the recognition site.

Table 1. Peptide Aldehyde Inhibitors of Calpain I.

Structure	IC_{50} (nM)
Aminoaldehyde	
Z-Leu-H	10,000
Dipeptides: P_2 Variation	
Z-β-Ala-Leu-H	>>10,000
Z-*t*-BuAla-Leu-H	140
Z-*t*-BuGly-Leu-H	4
Z-Cha-Leu-H	580
Z-Gln-Leu-H	370
Z-Ile-Leu-H	41
Z-Leu-Leu-H	8
Z-(*N*-Me)Leu-Leu-H	>10,000
Z-Nle-Leu-H	33
Z-Phe-Leu-H	230
Z-Ser-Leu-H	530
Z-Thr-Leu-H	190
Z-Trp-Leu-H	120
Z-Tyr-Leu-H	>1,000
Z-Val-Leu-H	29
Dipeptides: P_1 Variation	
Z-Leu-Abu-H	10
Z-Leu-Ala-H	120
Z-Leu-β-Ala-H	>>10,000
Z-Leu-β-Benzyl-β-Ala-H	>1,000
Z-Leu-*t*-BuGly-H	35
Z-Leu-Cha-H	11
Z-Leu-Gly-H	300
Z-Leu-His-H	15
Z-Leu-(*N*-Me)Leu-H	>1,000
Z-Leu-Met-H	33
Z-Leu-Nle-H	5
Z-Leu-Phe-H	10
Z-Leu-Tyr(*O*-Benzyl)-H	7
Z-Leu-Val-H	4
Dipeptides: Capping Group Variation	
Ac-Leu-Leu-H	130
FMOC-Leu-Leu-H	11
Maleyl-Leu-Leu-H	~10,000
(+)-Menthyloxycarbonyl-Leu-Leu-H	12
Ms-Leu-Leu-H	35
4-Nitro-Z-Leu-Leu-H	8
Phth-Leu-Leu-H	>1,000
Ts-Leu-Leu-H	10

Structure	IC_{50} (nM)
Tripeptides: P_3 & Capping Group Variation	
Ac-Ala-Leu-Leu-H	18
Ac-Leu-Leu-Leu-H	15
Ac-Ser(O-*t*-Bu)-Leu-Leu-H	37
Z-Ala-Leu-Leu-H	5
Z-Glu(*t*-Bu)-Leu-Leu-H	25
Z-Glu-Leu-Leu-H	40
Z-Leu-Leu-Leu-H	19
Z-Lys-Leu-Leu-H	20
Z-Phe-Leu-Leu-H	11
Z-Ser(O-*t*-Bu)-Leu-Leu-H	12
Z-Tic-Leu-Leu-H	15
Z-Tyr-Leu-Leu-H	8
Naphthyl-2-CO-Leu-Leu-Leu-H	10
Tripeptides: P_2 Variation	
Z-Leu-Ala-Leu-H	90
Z-Leu-Glu-Leu-H	1,200
Z-Leu-Gly-Leu-H	~10,000
Z-Leu-Lys-Leu-H	~10,000
Z-Leu-Phe-Leu-H	68
Z-Leu-Trp-Leu-H	45
Z-Val-Phe-Leu-H	31
Ac-Val-Phe-Leu-H	110
Tripeptides: P_1 Variation	
Ac-Leu-Leu-Arg-H	72
Ac-Leu-Leu-Glu(*t*-Bu)-H	1,100
Ac-Leu-Leu-Gly-H	~1,000
Ac-Leu-Leu-D-Leu-H	62
Ac-Leu-Leu-Lys(*t*-Boc)-H	33
Ac-Leu-Leu-Met-H	23
Ac-Leu-Leu-Nle-H	12
Ac-Leu-Leu-Phe-H	32
Ac-Leu-Leu-Trp-H	150
Tetrapeptides	
Z-Ala-Leu-Leu-Leu-H	6
Z-His-Leu-Leu-Leu-H	17

Various *N*-capping groups were examined in a series of dipeptide aldehydes while P_1 and P_2 were maintained as Leu. Both benzyloxycarbonyl (Z) and 4-nitrobenzyloxycarbonyl capping groups afford potent inhibitors (8 nM). Fluorenylmethoxycarbonyl (FMOC) gives similar potency (11 nM), suggesting that the S_3 binding pocket can accommodate spatially demanding groups. A benzylic substituent is not required; (+)-menthyloxycarbonyl also affords good potency (12 nM). Toluenesulfonyl (Ts) gives a potent inhibitor (10 nM), indicating that, as an alternative to the above carbamates, a sulfonamide is also well tolerated. Methanesulfonyl (Ms, 35 nM) and acetyl (Ac, 130 nM) afford weaker inhibitors, suggesting insufficient size for effective hydrophobic interactions with the S_3 pocket. Phthaloyl (Phth) and maleyl are poor capping groups, providing additional evidence that the P_2 N-H is required for hydrogen bonding to calpain I.

The P_3 binding site was examined with a series of acetyl-capped and benzyloxycarbonyl-capped tripeptide aldehydes in which P_1 and P_2 were maintained as Leu. The benzyloxycarbonyl, acetyl, and naphthylene-2-carbonyl capping groups attached to Leu-Leu-Leu-H give inhibitors of similar potency (10-19 nM). Human calpain I exhibits no stringent structural preferences at P_3: Hydrophobic (8-37 nM) and basic (20 nM) P_3 side chains as well as Ala (5-18 nM) are well tolerated, while Glu at P_3 gives slightly lower potency (40 nM). Interestingly, the best tripeptide aldehydes, and even tetrapeptide aldehydes, are no more potent than the best dipeptide aldehydes. In contrast, a protected aminoaldehyde, Z-Leu-H, inhibits very weakly (10,000 nM).

A series of benzyloxycarbonyl-capped tripeptide aldehydes was prepared in which P_2 was varied while P_1 and P_3 were held constant as Leu. Consistent with the dipeptide aldehyde series, Leu at P_2 affords the most potent inhibitor (19 nM). Phe (68 nM) and Trp (45 nM) at P_2 give moderate potency, however these tripeptides are somewhat more potent than the corresponding dipeptide aldehydes. This indicates that the additional amino acid residue of tripeptide aldehydes can compensate for non-optimal binding when the P_2 residue is other than Leu. In the tripeptide series, Ala at P_2 produces a modest inhibitor (90 nM), while Gly affords an extremely weak inhibitor (~10,000 nM), reemphasizing the importance of the P_2 aliphatic side chain. Glu at P_2 provides a poor inhibitor (1,200 nM), and P_2 Lys gives an extremely weak inhibitor (~10,000 nM), demonstrating that acidic or basic amino acids are not tolerated at P_2.

Finally, a series of acetyl capped tripeptides was prepared in which P_1 was varied, while P_2 and P_3 were maintained as Leu. The SAR reveals a preference for aliphatic side chains, and is generally consistent with the dipeptide aldehyde series. Nle at P_1 affords the most potent inhibitor (12 nM), while Leu, Met, and Phe give slightly lower potency (15-32 nM). Interestingly, an inhibitor containing D-Leu at P_1 still retains moderate potency (62 nM), but Gly does not bind well (~1,000 nM). Protonated Arg at P_1 also provides moderate potency (72 nM). Compared to Phe at P_1, Trp decreases potency nearly 5 times (150 nM), suggesting some

hindrance for planar aromatic side chains. In contrast, the highly sterically demanding, but flexible ε-*t*-Boc-Lys at P_1 affords moderate potency (33 nM). However, *O*-*t*-Bu-Glu is not tolerated at P_1 (1,100 nM).

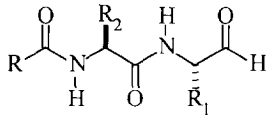
Discussion

Several significant trends emerge from the data. Dipeptide aldehydes generally possess greater or equal potency relative to homologous tripeptide and tetrapeptide aldehydes. This has important implications for the design of compounds for in vivo potency in which low molecular weight and a minimum number of peptide bonds are beneficial. The overall SAR is illustrated in Figure 1. In accord with previous investigations of peptide substrates[2,3] and peptide aldehyde inhibitors,[3-5] calpain I possesses a rather stringent requirement for Leu at P_2. In the dipeptide aldehyde series, *t*-BuGly at P_2 imparts greater potency than Leu, while other aliphatic amino acids, including Val, Nle, and Ile, decrease potency. Incorporation of aromatic and sterically demanding amino acids at P_2 gives even weaker inhibitors. The same trends are observed in the tripeptide series, but are less pronounced, indicating that favorable P_3 interactions can compensate for nonoptimal P_2 interactions. Thus the effects of the individual subsites are not totally independent. Similarly in the dipeptide series, Val at P_2 affords 4-fold lower potency than Leu when P_1 is Leu, but Val and Leu at P_2 give nearly equal potency when P_1 is Phe (Z-Val-Phe-H, IC_{50} = 11 nM).

Calpain I accepts a variety of aliphatic, aromatic, and basic amino acids at P_1 and (in tripeptide aldehydes) P_3, also in accord with previous examinations of substrates and inhibitors.[2-4] Consistent with previous reports, the amino group in dipeptide aldehydes can be capped with acyl, alkoxycarbonyl, or alkanesulfonyl groups, however, the P_2 N-H is required. Hydrogen bond donation from the P_1-P_2 and P_2-P_3 peptide bonds appears to be required, since *N*-methylation dramatically decreases binding affinity.

P_2: *t*-BuGly > Leu > Val ~ Nle > Ile > *t*-BuAla

Capping Group:
Z ~ 4-Nitro-Z ~ Ts ~ FMOC ~ (+)-Menthyloxy-CO > CH_3SO_2 > CH_3CO



P_1: Val ~ Nle ~ Tyr(*O*-Bn) > Leu ~ Abu ~ Phe ~ Cha > His

Figure 1. Subsite Preferences for Dipeptide Calpain I Inhibitors

Overall, by systematic variation of substituents, we have identified several new potent peptide aldehyde calpain I inhibitors. Z-Leu-Val-H, Z-Leu-Nle-H, Z-*t*-BuGly-Leu-H, and Z-Leu-(*O*-Benzyl)Tyr-H are the most

potent calpain I inhibitors yet reported. These inhibitors are up to 3 times more potent than Z-Val-Phe-H (MDL-28170, IC_{50} = 11 nM in our assay), which is reported to inhibit calpain I in vivo.[6] We anticipate that the subsite preferences of calpain I, determined with this series of di- and tripeptide aldehydes, will be useful for the design of peptide mimetic aldehydes as well as peptide-based inhibitors containing other enzyme-reactive groups.

Acknowledgments: The authors acknowledge Frederick W. Kauer and Coleen Yakow for assisting with chemical synthesis and thank Teresa M. O'Kane and Satish K. Mallya for measuring inhibitory potency of several peptide aldehydes. The authors also appreciate valuable discussions with James C. Kauer, John P. Mallamo, and Jeffry Vaught. We thank SmithKline Beecham for partial support of this research.

References and Notes

1. Mehdi, S. *Trends Biochem. Sci.* **1991**, *16,* 150. Wang, K. K. W. *Trends Pharmacol. Sci.* **1990,** *11,* 139. Wang, K. K. W.; Yuen, P.-W. *Trends Pharmacol. Sci.* **1994,** *15,* 412.

2. Takahashi, K. In *Intracellular Calcium-Dependent Proteolysis;* Mellgren, R. L.; Murachi, T., Eds; CRC: Boca Raton, 1990. Murachi, T.; Hatanaka, M.; Hamakubo, T. In *Neuropeptides and Their Peptidases*; Turner, A. J., Ed.; Ellis Horwood: New York, 1987; pp 202-228.

3. Sasaki, T.; Kikuchi, T.; Yumoto, N.; Yoshimura, N.; Murachi, T. *J. Biol. Chem.* **1984,** *259,* 12489.

4. Harris, A. L.; Gregory, J. S.; Maycock, A. L.; Graybill, T. L.; Osifo, K.; Schmidt, S. J.; Dolle, R. E. *Bioorg. Med. Chem. Lett.* **1995,** *5,* 393.

5. Kajiwara, Y.; Tsujinaka, T.; Sakon, M.; Kambayashi, J.; Ohshiro, T.; Murachi, T.; Mori, T. *Biochem. Int.* **1987,** *15,* 935. Sasaki, T.; Kishi, M.; Saito, M.; Tanaka, T.; Higuchi, N.; Kominami, E.; Katunuma, N.; Murachi, T. *J. Enz. Inhib.* **1990,** *3,* 195. Tsujinaka, T.; Kajiwara, Y.; Kambayashi, J.; Sakon, M.; Higuchi, N.; Tanaka, T.; Mori, T. *Biochem. Biophys. Res. Commun.* **1988,** *153,* 1201. Angelastro, M. R.; Mehdi, S.; Burkhart, J. P.; Peet, N. P.; Bey, P. *J. Med. Chem.* **1990,** *33,* 11. Saito, K.-I.; Nixon, R. A. *Neurochem. Res.* **1993,** *18,* 231. Woo, J.-T.; Sigeizumi, S.; Yamaguchi, K.; Sugimoto, K.; Kobori, T.; Tsuji, T.; Kondo, K. *Bioorg. Med. Chem. Lett.* **1995,** *5,* 1501. Saito, M.; Hashimoto, M.; Kodama, T.; Higuchi, N.; Tanaka, T.; Nomoto, K.; Murachi, T. *Agric. Biol. Chem.* **1987,** *51,* 861. Ishiura, S.; Murofushi, H.; Suz7uki, K.; Imahori, K. *J. Biochem.* **1978,** *84,* 225.

6. Hong, S.-C.; Goto, Y.; Lanzino, G.; Soleau, S.; Kassell, N. F.; Lee, K. S. *Stroke,* **1994,** *25,* 663.

7. Jurczak, J.; Golebiowski, A. *Chem. Rev.* **1989,** *89,* 149.

8. Ireland, R. E.; Liu, L. *J. Org. Chem.* **1993,** *58,* 2899.

9. Fehrentz, J.-A.; Castro, B. *Synthesis* **1983,** 676.

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. *Biochem. J.* **1996,** *314,* 511. Preliminary IC_{50}s determined with human erythrocyte-derived calpain I were consistent with IC_{50}s determined with recombinant human calpain I.

(*Received in USA* 18 *November* 1996; *accepted* 20 *January* 1997)