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Potent Affinity Labeling Peptide Inhibitors of Calpain[1)]

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We have employed a recently described affinity labeling approach using S-(3-nitro-2-pyridinesulfenyl) group (Npys) to devise unique inhibitors of the Ca^{++}-dependent thiol protease, calpain. Compounds with Npys group bound on the peptides with Leu-Leu or Val-Val sequences were found to be highly potent inhibitors of calpain.

Calpains (EC 3.4.24.17) are cytoplasmic Ca^{++} dependent proteases which play an important role in regulating various intracellular functions[2)] such as activation of protein kinase C, cleavage of membrane proteins, cytoskeletal modification and cleavage of surface proteins during platelet activation, and cleavage of epidermal growth factor.

A variety of inhibitors such as diazomethyl ketones, halomethyl ketones, and epoxides have been developed.[3)] However, there are potential problems with using these inhibitors as drugs, since they are relatively strong electrophilic reagents and may indiscriminately alkylate non-target enzymes and other biomolecules in vivo. For example, peptide epoxides are well known as irreversible inhibitors of cysteine proteases but they do not distinguish between calpain and cathepsins B, H and L.

To overcome these problems, we have developed a series of peptide disulfides which should eliminate non-specific alkylation and have the potential of being specific inhibitors of calpain. In a previous report,[4)] we have demonstrated the versatility of the affinity labeling approach by use of the Npys group for the design of inhibitors of thiol protease, cathepsin B. Sasaki *et al.*[5)] have reported that both calpain I, which requires μM Ca^{++}, and calpain II, which requires mM Ca^{++} for optimal enzymatic activity, exhibited similar specificity for amino acid residues that precede the peptide bond undergoing cleavage: Leu or Val at the P2 position and a rather bulky residue such as Met, Tyr, Lys or Arg at the P1 position. Considering the substrate specificity and the fact that Succinyl-Leu-Tyr-MCA is a good substrate for calpain, we postulated that Cys(Npys) residues could replace the P1-residue and can modify the SH function of the active site of calpain if inhibitors mimic the substrates.

Thus, we have designed various peptides which mimic substrates with Cys(Npys) at P1 and Leu, Ile, Val, Phe at the P2 and P3 positions as depicted in Fig. 1.

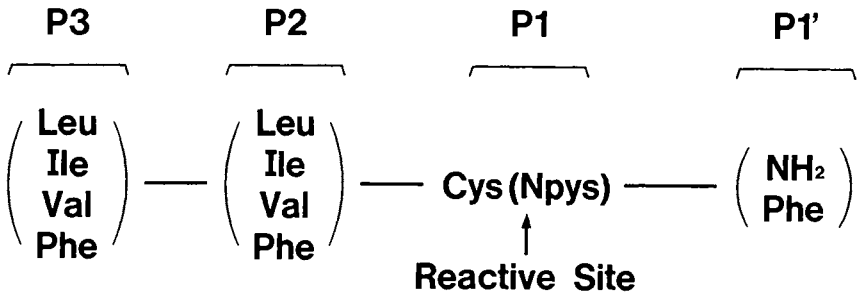

Fig. 1. Design of Calpain Inhibitors.

The synthetic peptide disulfide inhibitors and their potencies (IC_{50}) toward Purified human platelet calpain[6] are listed in Table 1.

Table 1. Synthetic Peptides and Their Inhibitory Potencies[a] Toward Calpain

Compounds[b]	Mp/°C (dec) ($[\alpha]_D^{22}$/° (*c* 0.2, MeOH))	Inhibitory potencies IC_{50}/M
TFA·H-Phe-Leu-Leu-Cys(Npys)-Phe-OH (1)	224-227 (-29.5)	2.20×10^{-5}
TFA·H-Leu-Phe-Cys(Npys)-NH_2 (2)	216-219 (-41.6)	9.18×10^{-6}
TFA·H-Leu-Leu-Cys(Npys)-NH_2 (3)	212-216 (-92.6)	1.80×10^{-7}
TFA·H-Ile-Ile-Cys(Npys)-NH_2 (4)	208-211 (-145.3)	1.42×10^{-5}
TFA·H-Val-Val-Cys(Npys)-NH_2 (5)	193-196 (-147.0)	1.85×10^{-5}
TFA·H-Leu-Nal-Cys(Npys)-NH_2 (6)	199-203 (-84.9)	6.84×10^{-6}
Ac-Ala-Gln-Val-Val-Ala-Gly-NH_2 (7)	283-286 (-22.3)	1.00×10^{-4}
TFA·H-Phe-Gln-Val-Val-Cys(Npys)-Gly-NH_2 (8)	230-234 (-75.6)	4.14×10^{-6}
2TFA·H-Arg-Leu-Phe-Cys(Npys)-NH_2 (9)	195-198 (-97.8)	6.50×10^{-5}

a) Inhibitory potencies were evaluated[7] toward the calpain which was dialyzed to remove DTT with Succinyl-Leu-Tyr-MCA as substrate : the maximum absorbance occured at 380 nm and emission maximum was observed at 450 nm. IC_{50} was calculated from the dose dependent curve for each inhibitor.

b) All the compounds were fully characterized by elemental and amino acid analyses.

All the peptides were prepared by the solid phase method using Boc-amino acids according to the general procedures of Stewart and Young.[8] In a typical experiment, starting from 2 g of benzhydrylamine resin (2% cross-linked polystyrene-divinylbenzene copolymer, containing 1.04 mmol of the amine component), the Boc derivatives of Cys(Npys), Leu, and Leu were coupled with 3 equiv. of dicyclohexylcarbodiimide and 6 equiv. of 1-hydroxybenzotriazole, using 3 equiv. of each amino acid in dimethylformamide. A coupling time of 2-24 h was used and the coupling was judged complete by the method of Kaiser *et al.*[9] The finished peptide resin was dried and treated with 20 ml of HF containing 2 ml of anisole for 30 min at 0 °C. After removel of the HF, the resin was washed with several portions of ethyl ether and the liberated peptide was extracted twice with 30 ml of TFA. The solvent was removed and the residue was treated with ethyl ether. The crude product was purified with Sephadex LH-20 equilibrated and eluted with MeOH or

by HPLC on a C-18 column using a linear gradient of 0.1% TFA in water to 50% acetonitrile in water containing 0.1% TFA. The yellow fractions showing a single spot on TLC were pooled and evaporated. The pure TFA·H-Leu-Leu-Cys(Npys)-NH_2·H_2O was obtained by precipitation from MeOH-ethyl ether; 303 mg, 47.4% yield from the initial benzhydrylamine resin: mp 212 - 216 °C (dec), $[\alpha]_D^{22}$ - 92.6° (*c* 0.2 MeOH). The amino acid ratios were: Leu, 2.0; $CySO_3H$, 0.87; NH_3, 1.28. Anal. Found: C, 41.86; H, 5.57; N, 13.37; S, 10.18; F, 9.31%. Calculated for $C_{22}H_{35}O_8N_6S_2F_3$: C, 41.76; H, 5.57; N, 13.29; S, 10.14; F, 9.01%.

The peptides with Cys(Npys) residue were found to be irreversible inhibitors. The release of NpySH from peptides 3 and 5 were detected by UV at 310 nm[10]: molar extinctions were 3.5×10^3 and 3.04×10^3, respectively. Inhibitory potencies of the peptides with Leu at P2 and P3 are higher than those with Ile or Val residues and H-Leu-Leu-Cys(Npys)-NH_2 (3) showed the highest potency and specificity toward calpain. The IC_{50} of the compounds 3 and 8 against calpain, cathepsin B,[11] and papain[11] were 1.8×10^{-7}M, 1.7×10^{-4}M, 1.9×10^{-4}M, and 4.1×10^{-6}M, 2.0×10^{-5}M, 4.0×10^{-5}M, respectively. They did not inhibit serine and acid proteases.

Ohkubo *et al.*[12] recently reported that the endogenous cysteine protease inhibitor (α_2TPI) in human plasma is identical with low molecular weight kininogen (LK) and it has the conserved common amino acid sequence of Gln-Val-Val-Ala-Gly which might be responsible for the inhibition of cysteine proteases. The similarity of the Val-Val part in this sequence to Val-Val or Lue-Leu sequences in compounds 3 and 5, stimulated us to design H-Phe-Gln-Val-Val-Cys(Npys)-Gly-NH_2 (7). We compared inhibitory potencies with Ac-Ala-Gln-Val-Val-Ala-Gly-NH_2 (8) which has no Cys(Npys) residue. Compound 7 was found not to inhibit calpain at 10^{-6}M but the substitution of Cys(Npys) for the Ala residue dramatically enhanced the potency (IC_{50} 8.2×10^{-6}M) as expected. Compound 8 was found to have the potential to inhibit thrombin-induced platelet aggregation.[13]

Thrombin-[13] and plasmin-induced[14] platelet aggregation have been shown to be mediated by the intracellularly activated calpain. High molecular weight kininogen (HK) inhibited platelet aggregation induced by the above plasma proteases. The nature and details of the mechanism by which HK inhibits thrombin- and plasmin-induced platelet aggregation are under investigation and will be reported in future. Domain 2 of HK also contains the consensus sequence, Gln-Val-Val-Ala-Gly.[15] Because of extensive sequence homologies between LK and HK, it has been suggested that the sequence Gln-Val-Val-Ala-Gly, present in domain 2 of HK, is also responsible for the inhibitory action of HK towards calpain. We recently found that H-Phe-Gln-Val-Val-Cys(Npys)-Gly-NH_2 (8) is a potent and specific inhibitor of thrombin- and plasmin-induced platelet aggregation.[16] Detailed investigations along these lines are still in progress and will be reported elsewhere.

The affinity labeling approach encompassing the use of Npys group bound to substrate or substrate-like peptides is novel for the design and synthesis of inhibitors for thiol proteases, e.g., calpain. These synthetic peptides show the promise of being specific inhibitors of thrombin- and plasmin-induced platelet aggregation. They may be helpful in designing protocols of thrombolytic therapy which could modulate the unwanted complications of restenosis and reocculsion.

References

1) The abbreviations used are those recommended by the IUPAC-IUB: J. Biol. Chem., 247, 977 (1972). The following abbreviations are also used: Boc = t-butyloxycarbonyl, TFA = Trifluoroacetic acid, MCA = 7-amino-4-methylcoumarin, Nal = (1'-Naphthyl)alanine.
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7) The calpain preparation (5-10 µl) was placed on a floating filter membrane[17] (Millipore type VMWP) over a buffer containing 50 mM Tris/HCl, pH 7.5 and 2.5 mM EDTA for 45-60 min, carefully removed and used for the inhibition studies. Aliquots (25 µl) of the enzyme (90 nm) and buffer or inhibitor were added to a cuvette at 25 °C containing 1 mM substrate in a buffer consisting of 60 mM Tris /HCl, pH 7.5, 2.5% DMSO and 5 mM $CaCl_2$.
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