
Developing selective inhibitors of calpain

Proteolysis is not confined to lysosomes, but also occurs in other organelles and in the cytosol^{1,2}. Calpain (Ca²⁺-activated neutral protease) is one of the major cytosolic proteases^{3,4}, and has been postulated to be involved in turnover of myofibrillar proteins⁵, protein kinase C activation⁶, cytoskeleton and cell membrane organization⁷, and modification of certain calmodulin-binding proteins⁸. It has a neutral pH optimum for activity, high specificity for its substrates, and an absolute dependence on Ca²⁺ for activity. Two isozymes have been identified, which have the same substrate specificity but differ in their affinity for Ca²⁺ (Ref. 9).

Calpain is a heterodimer, consisting of an 80 kDa catalytic subunit and a 29 kDa regulatory subunit. Molecular cloning has revealed that, in addition to the protease domain, the large subunit contains a calmodulin-like Ca²⁺-binding domain¹⁰. The small subunit contains a homologous Ca²⁺-binding domain¹¹ – hence the requirement of calpain for Ca²⁺. The catalytic site contains a cysteine residue (Cys108), which places calpain in the cysteine protease family. A histidine residue (His265) is in close spatial proximity¹⁰, and both these residues are involved in hydrolysis of the peptide bond.

Calpain inhibitors

In recent years, a large amount of research effort has been concentrated on identifying the physiological roles of calpain; experiments have often been carried out *in vivo*, and a wide variety of agents have been used to inhibit the action of the protease. Lack of selectivity of these agents for calpain has, however, posed major problems. For example, because the sulfhydryl group of Cys108 must be in its reduced form for the enzyme to be active, it is susceptible to thiol-reactive agents such as iodoacetic acid and *N*-ethylmaleimide (Table I), but these agents inhibit all cysteine proteases and indeed many other cellular proteins. Similar lack of selectivity is associated with Ca²⁺ chelators such as EDTA (although these have been used extensively to block calpain activity²⁻⁵), since these of course inhibit all Ca²⁺-dependent processes. The similarity between their Ca²⁺-binding domains means that calmodulin antagonists such as melittin and W7 also inhibit calpain¹², but with IC₅₀s 10–600 times higher than those for calmodulin.

There was thus a clear need for better inhibitors of calpain; in addition to having higher selectivity, these would also need to be membrane permeant for use *in vivo*. A basis for this search was

TABLE I. Inhibitors of calpain

Inhibitor	Remarks	IC ₅₀ (μM)		Commercial availability	Ref.
		calpain	papain		
Thiol-reactive agents (e.g. iodoacetic acid and N-ethylmaleimide)	also inhibit other cysteine proteases	5-20	5-20	yes	2-4
Leupeptin (R-Leu-Leu-Arg-H)	also inhibits other cysteine and serine proteases	0.2-1	0.05	yes	2-4
E-64	also inhibits other cysteine proteases	1-3	0.3	yes	25
cBz-Val-Phe-H	unknown specificity, more cell-penetrating than leupeptin	0.1	n.d.	yes	27
Calpeptin (cBz-Leu-nLeu-H)	more cell-penetrating than leupeptin	34-52	138	no	28
Ac-Leu-Leu-nLeu-H, Ac-Leu-Leu-nMet-H	improved selectivity	0.4-0.6	8	yes	
Leu-Leu-Phe-CH ₂ Cl	improved selectivity	0.2	2	no	29
Calpastatin	highly specific, tight binding	n.d.	n.i.	yes (crude)	21
Ca ²⁺ chelators (e.g. EGTA, EDTA, BAPTA)	also inactivate Ca ²⁺ -dependent processes and metallo-proteases	-		yes	2-4
Calmodulin antagonists (melittin, calmidazolium trifluoperazine and W7)	inhibit calmodulin-dependent enzymes or processes with 10-600-fold higher potency	2-251	n.i.	yes	12

Ac, acetyl (CH₃CHO); cBz, carbobenzoxy; nLeu, norleucine; nMet, normethionine; R, acetyl or propionyl; H, CHO; n.d., not determined; n.i., not inhibited.

provided by a model for the action of another cysteine protease, papain¹³. The active site of papain is proposed to accommodate synthetic substrates and inhibitors containing up to seven amino acid residues (referred to as P₄-P₃-P₂-P₁-P₁'-P₂'-P₃'), with the peptide bond for cleavage located between P₁ and P₁'. Complementary subsites on the protease are assumed to interact with amino acid side-chains and/or the polypeptide backbone of the substrate or inhibitor.

This model has been applied to other cysteine proteases; the amino acids at positions P₂, P₁ and P₁' are the most important¹³. A hydrophobic amino acid at position P₂ is a characteristic feature of substrates/inhibitors of all cysteine proteases, but calpain prefers Leu or Val (Ref. 14), whereas papain and most other cysteine proteases have a preference for Phe (Refs 13 and 15). At position P₁, calpain prefers Lys, Tyr, Arg or Met (Ref. 14; Fig. 1). These general rules do not always apply (for example, papain can accept Leu at P₂), but they can be used to design novel inhibitors which can then be tested against a variety of cysteine proteases (frequently papain) to determine their selectivity.

Peptide aldehydes

Probably the most widely used inhibitor of calpain is leupeptin - a microbial peptide aldehyde (Table I). On the basis of the above model, the second Leu and the Arg of leupeptin would fit into the positions P₂ and P₁, respectively, and the aldehyde end group would form a stable tetrahedral adduct with the sulfhydryl group of the active site cysteine to trap the enzyme. Complexing of leupeptin to calpain is Ca²⁺-dependent and is reversible upon removal of Ca²⁺ (Ref. 16).

However, leupeptin also suffers from a lack of selectivity: not only does it inhibit other cysteine proteases but it also acts as a serine protease inhibitor¹⁷. In fact, ironically, leupeptin was first discovered as an inhibitor of serine proteases¹⁸. It was relatively recently that leupeptin and antipain (another peptide aldehyde) were found to inhibit cysteine proteases (papain and cathepsin B)¹⁹. The IC₅₀ of leupeptin isolated from *Streptomyces roseus* is in the nanomolar range for most cysteine proteases (e.g. 0.05 μM for papain)²⁰ but about 200 μM for serine proteases¹⁹. However, leupeptin has an intermediate IC₅₀ (about 0.2-1 μM) for calpain^{21,22}; thus it has about ten times lower

affinity for calpain than for papain. This must be taken into account when interpreting results because a large variety of serine proteases, and cysteine proteases other than calpain [including the multifunctional protease (proteosome) and the ATP-dependent protease^{1,23}] are present in the cytosol, lysosomes and other organelles. Nevertheless, leupeptin has been used, with some success, to block degradation of protein kinase C *in vivo*²⁴.

Epoxy-succinyl peptides

E-64, which is isolated from *Aspergillus japonicus*, is a member of another class of microbial peptide inhibitors of calpain (epoxy-succinyl peptides)²⁵ (Table I). In the presence of Ca²⁺, E-64 irreversibly inhibits calpain (but also other cysteine proteases) by alkylating the sulfhydryl group of the catalytic site Cys to form thioethers²⁵. Papain, cathepsin B, cathepsin L and calpain are rapidly inactivated while streptococcal cysteine protease is only slowly inactivated and clostripain is not inhibited at all¹⁵. E-64 does not inhibit serine proteases, because they lack an active site Cys. While the membrane permeance of E-64 and E-64-c is relatively poor, E-64-d, a derivative of E-64-c (Fig. 1), is membrane permeant²⁶.

Improvements in selectivity and permeance

Rational design of small peptides is now leading to improvements in calpain inhibitors. For example, the presence of the positively charged arginine residue means that the permeance of leupeptin is low: 100 μM of leupeptin (in the medium) was unable to block calpain-mediated proteolysis of membrane proteins in intact rat erythrocytes, while the more hydrophobic peptide aldehyde, cBz-Val-Phe-H (Sigma) was more active (IC₅₀ 1 μM) (Table I). By contrast, leupeptin and cBz-Val-Phe-H had identical IC₅₀s (0.1 μM) *in vitro*²⁷. The specificity of cBz-Val-Phe-H towards calpain has not been studied²⁷, but the presence of Val in position P₂ indicates that it is likely to inhibit other cysteine proteases.

Another membrane-permeant peptide aldehyde, calpeptin (cBz-Leu-nLeu-H), has improved selec-

tivity for calpain over papain (Table I) *in vitro*. It has been used successfully to pretreat intact platelets and to inhibit calpain-mediated proteolysis of platelet proteins (IC_{50} 29 μM)²⁸. Calpeptin is not yet commercially available. The peptide aldehydes Ac-Leu-Leu-nLeu-H and Ac-Leu-Leu-nMet-H (Calbiochem) also have selectivity for calpain over papain (T. Murachi, unpublished). Both compounds have a Leu in the P₂ position.

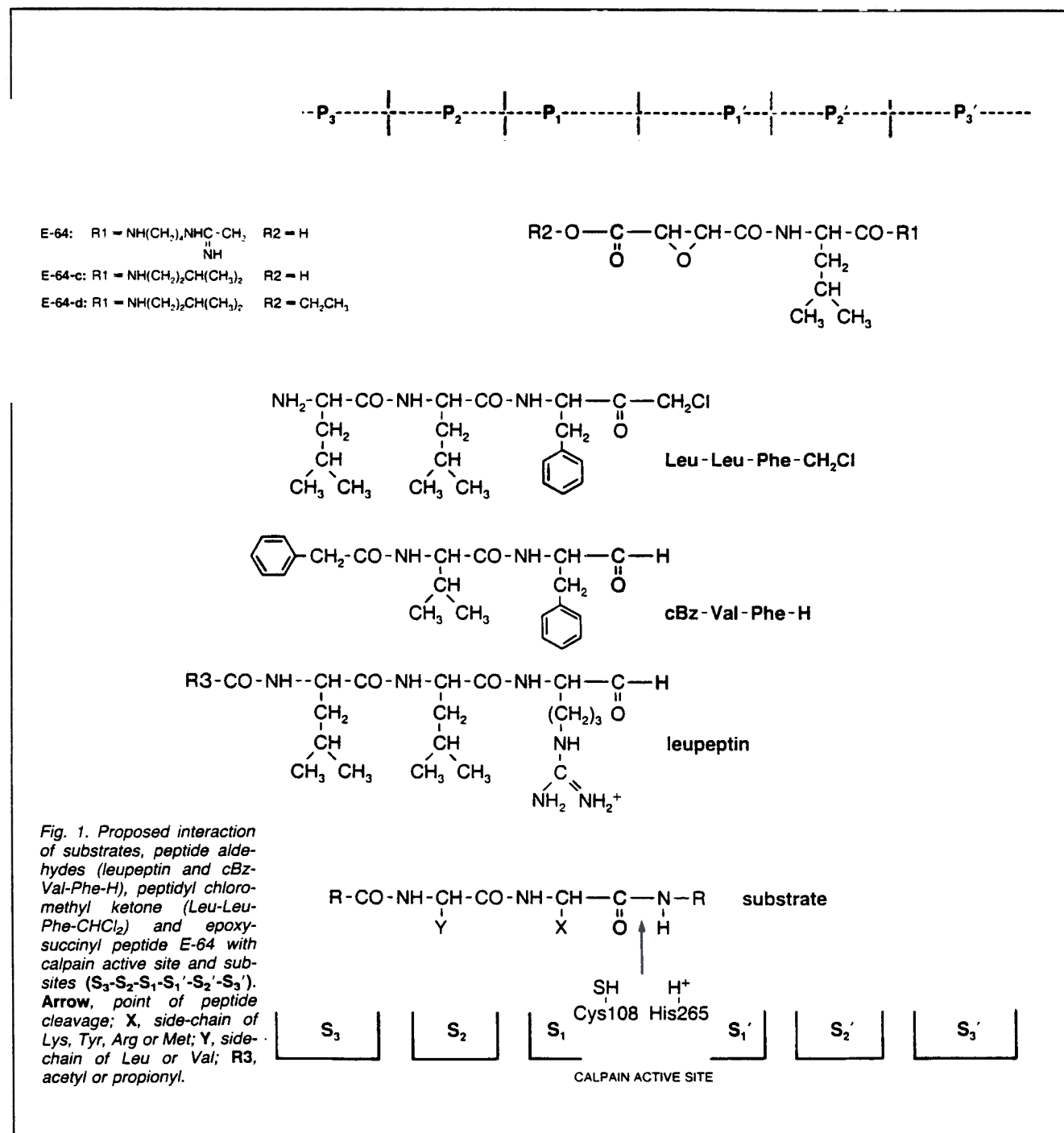
Murachi and colleagues²⁹ have designed a calpain inhibitor (Leu-

Leu-Phe-CH₂Cl), which belongs to the peptidyl chloromethyl ketone family of protease inhibitors. These compounds alkylate the sulfhydryl group on the active site Cys in cysteine proteases. Leu-Leu-Phe-CH₂Cl appears to show some selectivity for calpain (IC_{50} 0.2 μM) over papain (IC_{50} 2 μM). The membrane permeance of this compound has not been reported.

Endogenous inhibitor calpastatin

Calpastatin is an endogenous calpain inhibitor protein⁵ (Table

I). It is a homotetramer in the absence of Ca²⁺, and in the presence of Ca²⁺ it dissociates into active monomers. Two types of calpastatin exist: the liver-type is ~ 68 kDa and contains four repeated domains; and the erythrocyte-type is 46 kDa and has three repeated domains⁵. Each repeat binds and inhibits one molecule of calpain⁵. The binding between calpastatin and calpain is Ca²⁺-dependent and is reversed upon removal of Ca²⁺; once bound to calpastatin, calpain is incapable of autolysis or proteolysis of



substrates. Calpastatin is highly specific for calpain, and does not inhibit other proteases⁵.

Calpastatin is heat stable and relatively easy to purify in large quantities^{19,30}. Indeed, rabbit skeletal muscle calpastatin is available commercially (Sigma) as a crude protein mixture. Further purification before use would be desirable. The large size of calpastatin impedes cell penetration, but it is possible to microinject calpastatin into culture cells of interest³¹.

Recently, Murachi and colleagues have demonstrated that a synthetic oligopeptide (27 residues) corresponding to a conserved region of calpastatin inhibits calpain activity³². Defining and modifying the shortest inhibitory calpastatin peptide will provide an alternative approach to that based on the substrate specificity of calpain.

□ □ □

At present, calpain activity can best be blocked *in vivo* by the use of Ca²⁺ chelator in combination with at least one other calpain inhibitor (e.g. E-64, cBz-Val-Phe-H or calpastatin). However, until selective inhibitors have been designed, the potential effects of the widely used 'calpain inhibitors' on other cytosolic or lysosomal proteases must always be borne in mind.

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BAPTA: bis-(*o*-aminophenoxy)-ethane-*N, N, N', N'*-tetraacetic acid
W7: *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride