Drugs of the Future 1998, 23(11): 1217-1225

CCC: 0377-8282/98

Review Article

Recent advances in the development of calpain I inhibitors

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Introduction

Calcium-activated neutral proteases (calpains) comprise a family of intracellular cysteine proteases. Two major forms of calpain, calpain I (μ-calpain, a low Ca^{2+}-requiring form) and calpain II (m-calpain, a high Ca^{2+}-requiring form), respectively, are ubiquitously expressed in mammalian tissues (1). Calpain is a heterodimer. Each isozyme is composed of an 80 kDa large catalytic subunit and a 30 kDa small regulatory subunit (2). While the 80 kDa subunit is unique to each form, the 30 kDa subunit is common to both of them. Though literature reports suggest that calpain exists primarily as an inactive proenzyme that requires autolytic cleavage for activation, there is now increasing evidence that nonautolyzed calpain also might be a physiologically active form (3). While calpain II is the predominant form in many tissues, calpain I is thought to be the predominant form activated during pathological conditions of nervous tissues. However, in a recent paper, Brorson *et al.* indicated that calpain II might also be involved in protein breakdown in hippocampal neurons (4). It should, however, be noted that in recent years a growing number of distinct homologues to the protease domain of calpain I and II from diverse organisms have come to light (5). Thus, p94 (also known as calpain 3) expressed in mammalian skeletal muscle, has been implicated in limb-girdle muscular dystrophy type 2A. The possible role of this enzyme in cleaving specifically the skeletal muscle ryanodine receptor/Ca^{2+} release channel has also been documented (6). Tra-3, a calpain homologue in nematodes, plays a role in the sex determination cascade during early development. In fungi, PalB, a key gene product, has been identified as a calpain homologue.

Calpain I has been implicated in many nervous system disorders including stroke, Alzheimer's disease, amyotrophy, motor neuron damage and muscular dystrophy. Thus, its inhibition has become an important pharmacological goal (7). In recent years, a number of calpain I inhibitors have been reported in the literature. A majority of these are substrate-based inhibitors containing enzyme-reactive groups. These compounds can be categorized in two ways: reversible inhibitors that inactivate an enzyme in a transient manner and irreversible inhibitors that permanently inactivate an enzyme. Reversible inhibitors include peptidyl aldehydes and activated ketones, *e.g.*, α-keto esters, α-keto acids and α-keto amides. Irreversible inhibitors include peptidyl haloketones, diazoketones, epoxysuccinyls and other derivatives. In this review, we will first describe the recent developments in the enzyme reactive group area. This will be followed by reports of the development of novel peptidomimetic and nonpeptidic inhibitors, respectively.

Exploration of enzyme reactive groups

Peptidyl aldehydes

Various peptide aldehyde inhibitors of calpain I have been reported in the literature (8). These include calpain inhibitor I, calpeptin, leupeptin and Cbz-Val-Phe-H (MDL-28170) (9). However, much of the data reported in the literature was generated with calpains from different sources under different assay conditions. In 1995, Harris and coworkers, disclosing a sensitive, continuous fluorogenic assay, reported a limited set of dipeptide aldehyde inhibitors of human erythrocyte calpain I (10). Later, Iqbal *et al.* undertook a comprehensive study to define the subsite requirement for this class of inhibitor against human recombinant calpain I (rh calpain I) (11).

In general, dipeptide aldehydes (**1**, Fig. 1) possess equal (or sometimes greater) potency relative to tripeptide and tetrapeptide aldehydes. This observation was useful for the design of peptidomimetic inhibitors of calpain I (see below). While calpain I tolerates a variety of aliphatic and aromatic amino acids at P_1, Leu and Val at P_2 are favored (12). Interestingly, previously unrecognized, *tert*-butylgly is also well tolerated at the P_2 site. Among various *N*-terminal capping groups explored, Cbz, 4-nitro-Cbz, tosyl and Fmoc are favored.

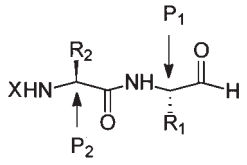

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Fig. 1.

The neuroprotective effect of membrane-permeable Cbz-Val-Phe-H (MDL-28170) in various models of neurodegeneration has been well documented. In an *in vitro* model of neurotoxicity, Caner *et al.* exposed cerebellar slices from young rats to AMPA, a potent glutamate receptor agonist (13). This produced damage to 83% of cerebellar Purkinje cells. However, the damage was confined to only 23.6% of Purkinje cells when the slices were treated with Cbz-Val-Phe-H and AMPA. Brorson *et al.* reported that Cbz-Val-Phe-H blocked the neurotoxic effects of NMDA in cultured hippocampal neurons and of kainate in cultured cerebellar neurons (4). The inhibitor also limited the toxicity, even when applied for up to 1 h after the onset of the toxic exposure. Similarly, Rami *et al.* demonstrated that Cbz-Val-Phe-H protected hippocampal neurons from glutamate-induced toxicity (14). Hong *et al.* examined the effect of Cbz-Val-Phe-H on the pathological outcome after transient focal cerebral ischemia in rats (15). Ischemia was induced by occluding the left middle cerebral artery (MCA) and both common carotid arteries for 3 h followed by reperfusion. It was reported that rats treated with Cbz-Val-Phe-H exhibited significantly smaller volumes of cerebral infarction than vehicle-treated or saline-treated control animals. Cumulative doses of 30 or 60 mg/kg i.v. of the inhibitor were effective in reducing infarction, edema and calcium-activated proteolysis. In an accompanying commentary, the guest editor pointed out that "...one important observation resulting from this study needs to be emphasized: the neuronal protective effect of this compound appears to be unrelated to cerebral blood flow, brain temperature, or blood-brain barrier permeability property, since the proteolytic response to postdecapitation ischemia is also reduced by this calpain inhibitor" (16). In a recent study, Markgraf and coworkers, employing Cbz-Val-Phe-H, reported a 6-h window of opportunity for calpain inhibition in a reversible focal cerebral ischemia model in rats (17). The MCA occlusion was accomplished by advancing a monofilament through the internal carotid artery to the origin of the MCA. The authors reported that the inhibitor reduced infarct volume when therapy was delayed for 0.5, 3, 4 and 6 h after the initiation of damage. However, the protective effect was lost after an 8-h delay of treatment.

Peptidyl α-keto esters

Angelastro *et al.* reported Cbz-Val-Phe-$COOCH_3$ (K_i = 0.4 μM) and Cbz-Val-Phe-$COOCH_2CH_3$ (K_i = 0.6 μM) to be inhibitors of purified calpain from chicken gizzard (18). It was shown that deletion of the P_2 amino acid had a significant negative effect on potency (*e.g.*, Cbz-Phe-$COOCH_2CH_3$ K_i = 92 μM). The authors disclosed that the α-diketone, Cbz-Val-Phe-$COCH_3$, also inhibited calpain (K_i = 0.7 μM). Later, Li *et al.* reported an extensive SAR study of peptidyl α-keto ester inhibitors of human erythrocyte calpain I and other cysteine proteases (19). In the series Cbz-Leu-D,L-AA-COOEt, calpain I preferred Met, Nva and Phe over 4-Cl-Phe, Abu and Nle; however, this trend was reversed for calpain II. Exploration of the nature of the *N*-terminal group on the potency of a series of RCO-Leu-D,L-Abu-COOEt compounds indicated that Ph_2CHCO-Leu-D,L-Abu-COOEt was a 45-fold more potent inhibitor of calpain I than Cbz-Leu-D,L-Abu-COOEt. However, variation of the ester moiety in the series Cbz-Leu-D,L-Abu-COOR produced a less pronounced effect. Thus, Cbz-Leu-D,L-Abu-COO-nBu was 2.5 times more potent against calpain I than the parent Cbz-Leu-D,L-Abu-COOEt. The authors reported that, in general, the inhibitors were 3- to 190-fold selective for calpain I over cathepsin B, a related cysteine protease, the most selective being Cbz-Leu-D,L-Phe-COOEt (calpain I K_i = 1.8 μM; cathepsin B K_i = 340 μM). A number of α-keto esters were evaluated for membrane penetration in a rat platelet assay: $Ph(CH_2)_2CO$-Leu-D,L-Abu-COOEt (IC_{50} = 20 μM) and $PhOCH(C_2H_5)CO$-Leu-D,L-Abu-COOEt (IC_{50} = 22 μM) were the two most active compounds in this assay. Cbz-Leu-D,L-Phe-COOEt, the most selective analog, had an IC_{50} of 200 μM in this assay.

Peptidyl α-keto amides

Rapid degradation of the previously mentioned α-keto esters (probably by plasma esterases) during *in vivo* studies motivated Li *et al.* to explore more stable α-keto amides (19). In the series Cbz-Leu-D,L-AA-CONHEt, compounds with P_1-Abu, -Nva or -Phe were equipotent. However, addition of alkyl or arylalkyl substituents to the nitrogen of the α-keto amide moiety in Cbz-Leu-D,L-Phe-CONHR resulted in improved potency; thus, the most potent compound of the series was Cbz-Leu-D,L-Phe-$CONH(CH_2)_2Ph$ (K_i = 0.052 μM). Interestingly, *N,N*-disubstituted α-keto amides were much less active than the *N*-monosubstituted α-keto amides: Cbz-Leu-D,L-Phe-$CONEt_2$ was 380-fold less potent than Cbz-Leu-D,L-Phe-CONHEt. This led to the hypothesis that a hydrogen bond acceptor in the S_1' subsite of the enzyme may interact with the *N*-H of the keto amide moiety. Cbz-Leu-D,L-Abu-$CONH(CH_2)_8CH_3$ was the most selective member of the series for calpain I (K_i = 0.12 μM) over cathepsin B (K_i = 150 μM). A number of α-keto amides were also evaluated for membrane penetration in the rat platelet assay: Cbz-Leu-D,L-Phe-CONHEt (IC_{50} = 22 μM) and Cbz-Leu-D,L-Phe-CONH-*i*Bu (IC_{50} = 22 μM) were the two most potent compounds in this assay.

In a subsequent publication, Li *et al.* expanded their work on α-keto amides (20). In the general structure R_1-Leu-D,L-AA-$CONHR_2$, they explored 10 different R_1s, 3

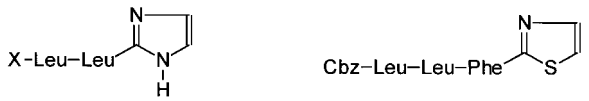
different AAs and 44 different R_2s. The best calpain I inhibitor in this study was Cbz-Leu-D,L-Nva-CONH-CH_2-2-pyridyl (K_i = 0.019 μM), which was approximately 40-fold more selective for calpain I over cathepsin B. The most selective compound, however, was Cbz-Leu-D,L-Phe-CONH$(CH_2)_3$-4-morpholinyl (108 times more selective for calpain I over cathepsin B). Through examination of the structures of the potent inhibitors (containing 2-pyridyl, -CH_2CHOHPh, *etc.* in the S' sites), it was postulated that there might be additional H-bonding sites (donors or acceptors) in the S' subsites (S_2', S_3', *etc.*) of the enzyme; however, many analogs lacking this feature were also very active. In the rat platelet membrane permeability assay, Cbz-Leu-D,L-Nva-CONH-$(CH_2)_3$-4-morpholinyl displayed the most potency (IC_{50} = 18 μM).

Harbeson *et al.* reported a stereospecific synthesis of L,L-dipeptidyl α-keto amides (21). The potent inhibition of porcine calpain I by the L,L diastereomers, combined with poor inhibition by the L,D diastereomers, revealed the importance of all L-stereochemistry in potent inhibitors (cf. Cbz-L-Leu-L-Phe-CONHEt with a K_i of 36 nM *vs.* Cbz-L-Leu-D-Phe-CONHEt with a K_i of >1500 nM). The synthetic method allowed the authors to incorporate various solubilizing groups at the C- and *N*-termini, maintaining the potency. On stereochemical integrity, the authors noted that under general base conditions, epimerization at P_1 took place rapidly; however, optical purity could be maintained in unbuffered or slightly acidic conditions.

Two of the peptidyl α-keto amides from the above studies were also evaluated for their effectiveness in an animal model of ischemia. Focal ischemia was created using a variation of the MCA occlusion model. In one experiment, diastereomerically pure Cbz-Leu-Abu-CONHEt (AK275) was perfused directly onto the infarcted cortical region (22). This was done with the intent of reducing or eliminating various pharmacokinetic, hemodynamic and other potentially confounding variables that might complicate interpretation of any drug effect. In another experiment, Cbz-Leu-Abu-CONH$(CH_2)_3$-4-morpholinyl (AK295) was infused through the internal carotid artery (23). After the customary delay, the animals were sacrificed and the infarct volume measured. AK275 was able to reduce the infarct volume by 75%, while AK295 reduced the volume by 32% (however, the dosing regimen was different). AK295 has also been reported to attenuate motor and cognitive deficits in a rat model of brain injury (24).

Peptidyl α-keto acids

Li *et al.* reported a pair of potent peptidyl α-keto acids (19). Cbz-Leu-D,L-Phe-COOH (K_i = 0.0085 μM) was 212-fold more potent than the corresponding α-keto ester Cbz-Leu-D,L-Phe-COOEt (K_i = 1.8 μM). In a similar manner, Cbz-Leu-D,L-Abu-COOH (K_i = 0.075 μM) was 60-fold more potent than Cbz-Leu-D,L-Abu-COOEt (K_i = 4.5 μM). Cbz-Leu-D,L-Phe-COOH was also 529-fold more selective for calpain I over cathepsin B. However, ionization of both α-keto acids made them poor inhibitors in the rat platelet membrane permeability assay; the IC_{50} for each compound was 100 μM.



2) X = *t*Boc **3**) X = Cbz **4**

Fig. 2.

Peptidyl heterocycles

Tao *et al.* explored a series of peptidyl heterocycles (designed to mimic peptide ketoamides and ketoacids) as potential inhibitors of rh calpain I (25). Previously, peptidyl heterocycles were reported as potent inhibitors of the serine protease elastase (26-28), prolyl endopeptidase (29, 30) and thrombin (31-33).

Among various dipeptidyl heterocycles examined, *tert*-Boc-Leu-Leu-imidazole (**2**) exhibited moderate potency (77% inhibition at 10 μM, Fig. 2). However, replacement of the *N*-terminal *tert*- Boc by Cbz (**3**) abolished the activity. Similarly, protection of the NH moiety of the imidazole nucleus in compound **2** by the trimethylsilylethoxymethyl (SEM) group produced an inactive compound (up to 10 μM). In the thiazole series, tripeptidyl Cbz-Leu-Leu-Phe-thiazole (**4**) inhibited the enzyme (54% inhibition at 10 μM). In the tetrazole series, Cbz-Leu-Leu-tetrazole showed marginal activity (11% inhibition at 10 μM); this compound was designed to mimic the corresponding alpha-keto acid analog, Cbz-Leu-Leu-COOH, a potent rh calpain I inhibitor (34).

Peptidyl phosphonates, phosphinates and phosphine oxides

In their continuing search for novel enzyme reactive groups, Tao *et al.* also explored a series of dipeptidyl α-ketophosphorous analogs as potential inhibitors of rh calpain I (35). Among various compounds tested, phosphonate Cbz-Leu-Leu-P(O)$(OCH_3)_2$ (IC_{50} = 0.43 μM), phosphinate Cbz-Leu-Leu-P(O)(Ph)OEt (IC_{50} = 0.37 μM) and phosphine oxide Cbz-Leu-Leu-P(O)$(C_6H_4$-p-Cl$)_2$ (IC_{50} = 0.35 μM) displayed inhibitory activity against rh calpain I.

Peptidyl fluoromethyl ketones

Peptidyl fluoromethyl ketones and their inhibitory activity against human cathepsin B were first reported in the literature by Rasnick (36). Later, Shaw *et al.* (37) reported a dipeptide, Cbz-Leu-D,L-Tyr-CH_2F to be an inactivator of chicken gizzard calpain II (rate of inactiva-

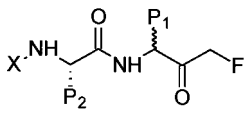


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Fig. 3.

tion = 17,000 M^{-1} s^{-1}). Chatterjee *et al.* systematically explored a series of dipeptide fluoromethyl ketones (compound **5**, as a diastereomeric mixture at P_1, Fig. 3) as potential rh calpain I inhibitors (38, 39). It was shown that at P_1, Phe was preferred over Abu and Ser, respectively. Interestingly, protection of the hydroxyl group of the P_1-Ser residue as a tetrahydropyranyl (THP) ether moiety generated an approximately 5-fold more potent compound. Thus, calpain I preferred a hydrophobic group at the P_1 site of this class of molecules. Previous studies indicated that calpain accepts Leu or Val at P_2; however, in this series, an inhibitor with P_2-Leu displayed 4 times more potency than an inhibitor with P_2-Val.

The nature of the *N*-terminal capping group (X) played a significant role in the potency of this series of compounds. While Cbz, *tert*-Boc, morpholinosulfonyl and benzylaminocarbonyl were all well tolerated, Cbz was preferred. The lack of any capping group, resulting in weak activity, indicated the importance of an *N*-terminal capping group. Replacement of the OCH_2 moiety in the Cbz capping group with the CH_2CH_2 moiety generated a less active analog, revealing that an additional hetero atom in the region might be involved in an energetically beneficial binding. Interestingly, a tetrahydroisoquinolyl capping group with Leu-D,L-Phe backbone generated a very potent dipeptide fluoromethyl ketone inhibitor (rate of inactivation = 276,000 M^{-1} s^{-1}). This compound displayed 37- and 6-fold more selectivity for calpain I over cathepsin B and cathepsin L, respectively. It also inhibited calpain I in a human leukemic T cell line (IC_{50} = 0.2 μM).

Peptidyl chloromethyl ketones and (acyloxy)methyl ketones

Harris *et al.* reported a series of di- and tripeptide chloromethyl ketone and (acyl)aryloxymethyl ketone inactivators of human erythrocyte calpain I (10). In general, the P_2-Leu/Val and P_1-Phe/Tyr residues displayed greater activity than other amino acids at these positions. However, the authors noted the profound influence of leaving group structure on potency which could override calpain's P_1-P_2 specificity preferences. Thus, dipeptides Cbz-Leu-Gly-CH_2Cl and Cbz-Leu-Gly-CH_2OCO-2,5-Cl_2-3-SO_2-morpholine-Ph, containing P_1-Gly, displayed inactivation rates of 31,000 M^{-1} s^{-1} and 23,000 M^{-1} s^{-1}, respectively. The tripeptide Cbz-D-Ala-Leu-Phe-CH_2OCO-2,6-F_2-Ph displayed >300- and >100-fold more selectivity for calpain I over cathepsin B and cathepsin L, respectively.

Peptidyl benzotriazol-1-yl-oxymethyl ketones and benzotriazin-4-one-3-yl-oxymethyl ketones

A series of dipeptidyl benzotriazol-1-yl-oxymethyl ketone inactivators of rh calpain I were reported by Wells *et al.* (40). The representative example, compound **6** (rate of inactivation = 320,000 M^{-1} s^{-1}), is shown in Figure 4. SAR studies revealed that in this series of compounds, P_1-Leu was slightly preferred over P_1-Phe. Substitutions around the benzene ring revealed the importance of steric factors over electronic factors. Modification of the benzotriazole to imidazole or benzimidazole congeners produced less active compounds revealing the uniqueness of this ring system. Incorporation of a nitrogen into the benzene ring of the benzotriazole was tolerated depending on its position in the ring.

In an extension of the above series, Wells *et al.* also described a series of peptidyl benzotriazin-4-one-3-yl-oxymethyl ketones (general structure **7**). P_1-Phe was favored in the benzotriazinone series. In this series also, substitutions around the benzene ring revealed the importance of steric factors over electronic factors.

Peptidomimetic inhibitors

Ketomethylene (-$COCH_2$-) containing inhibitors

Described earlier, both tripeptide and dipeptide aldehydes are potent inhibitors of calpain I. Chatterjee and coworkers, therefore, postulated that sufficient binding energy is obtained with occupancy of the S_1 and S_2 subsites of the enzyme. Dolle *et al.*, however, claimed that for a peptidic inhibitor of calpain I (a member of the papain superfamily), the P_2-NH moiety is critical for hydrogen bonding (41). Takahashi, on the other hand, commenting on preferred substrates, noted that "...an amino acid with an aromatic or a bulky aliphatic side chain at the P_3 position may to some extent increase the susceptibility of the scissile bond to calpain" (42). In order to probe the importance of the P_2-NH moiety in a tripeptide or a dipeptide inhibitor of calpain I, Chatterjee *et al.* replaced the P_3-P_2 amide bond in the tripeptide inhibitor, or the carbamoyl/acyl moiety in the dipeptide inhibitor, with a ketomethylene (-$COCH_2$-) moiety. In designing their target molecules, they maintained an isobutyl group at the pseudo-P_2 site to mimic the P_2-Leu of the corresponding peptidic inhibitors but incorporated an aromatic moiety in the P_3 region (43, 44).

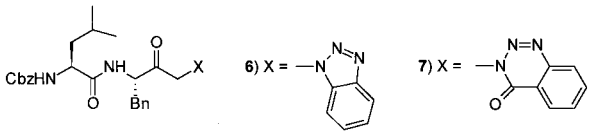
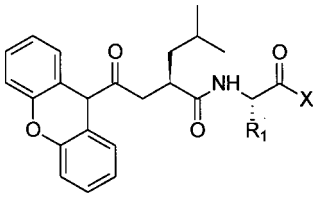


Fig. 4.



8) R_1 = iBu, X = H

9) R_1 = iBu, X = CONHEt

10) R_1 = Bn, X = CH_2F

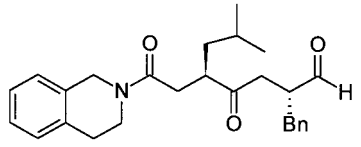
Fig. 5.

Systematic SAR studies revealed that attachment of a sterically demanding aromatic group to the carbon atom of the carbonyl group of the ketomethylene moiety would be beneficial. This gave rise to tricyclic xanthene containing aldehyde (**8**, IC_{50} = 25 nM), α-ketocarboxamide (**9**, IC_{50} = 130 nM) and fluoromethyl ketone (**10**, k_{obs} = 76,000 M^{-1} s^{-1}) inhibitors, respectively (Fig. 5). Compounds **8**, **9** and **10** preferred calpain I by >17-, approx. 9- and 76-fold, respectively, over cathepsin B. The compounds were cell-permeable and inhibited calpain I in a human leukemic T cell line (IC_{50}s = <10 μM).

In order to explore the effect of stereochemistry at the pseudo P_2 site, diastereomeric ketomethylene compounds **11** and **12** (Fig. 6) were generated (44); the stereochemistry was assigned based on the comparison of inhibitory potency of **11** and **12** with that of the reference aldehyde Cbz-Val-Phe-H. Compound **11**, with (*R*)-stereochemistry (which corresponds to the (*S*)-stereochemistry in the P_2 position of a dipeptidic inhibitor) was about 36 times more potent than the diastereomeric compound **12** with (*S*)-stereochemistry indicating the preferred stereochemical requirement of calpain I for the pseudo P_2 site of this class of inhibitor.

Carbamethylene (-CH_2CH_2-) containing inhibitors

Chatterjee *et al.* also incorporated the -CH_2CH_2- moiety between P_2 and P_3 to generate a series of peptidomimetic carbamethylene inhibitors of rh calpain I (44, 45). In this case, a fused biphenyl system, derived from



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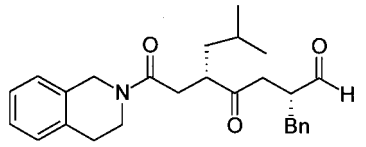
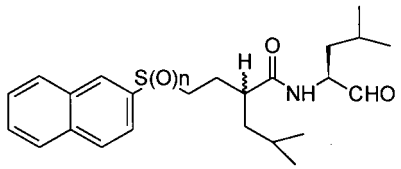


12

Fig. 6.



13 = ····H n = 2 **14** = —H n = 2

15 = —H n = 0 **16** = —H n = 1

Fig. 7.

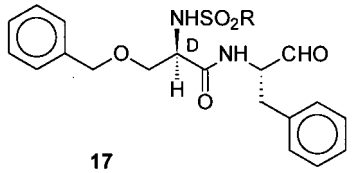


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Fig. 8.

2-naphthalenethiol, was used as a spanning P_3 moiety (Fig. 7).

In the sulfonyl series, between the diastereomeric pair, inhibitor **14** (IC_{50} = 50 nM) was 10 times more potent than inhibitor **13** (IC_{50} = 500 nM), indicating the preferred stereochemical requirement of rh calpain I for the psuedo P_2 site of this class of inhibitor also. Both the sulfoxy (as the diastereomeric mixture, epimeric at the sulfoxide center) analog **16** (IC_{50} = 30 nM) and the sulfonyl analog **14** were more potent than the corresponding thio analog **15** (IC_{50} = 75 nM). However, the selectivity of the compounds for calpain I over cathepsin B was modest (2- to 3-fold).

D-Amino acid derived inhibitors

Activity of peptidomimetic ketomethylene and carbamethylene containing compounds revealed that the NH at the P_2 site of a potent dipeptide inhibitor could effectively be replaced by a CH_2, provided an aromatic moiety was employed in the P_3 region. Chatterjee *et al.* reasoned that the requirement of calpain I for an isobutyl group (from leucine) or an isopropyl group (from valine) from the P_2 site of an L,L-dipeptide inhibitor might be steric in nature; either of these moieties could occupy the same pocket of the enzyme's S_2 subsite. In designing their next generation target molecules, the authors decided to replace the P_2-isopropyl group in the known potent calpain I inhibitor, Cbz-Val-Phe-H by a sulfonamido group. At the same time, they incorporated an aromatic moiety in the P_3 region attached by a spacer to the P_2 site. This generated a series of P_2-D-Ser(Bn)-derived inhibitors (**17**, Fig. 8); the parent compound (R = Me) was equipotent to the reference compound Cbz-Val-Phe-H (46).

SAR studies around the P_1 site revealed that Phe was preferred over Abu and Leu. The S_1 pocket of the enzyme also tolerated large hydrophobic groups such as

Lys(SO_2Ph) and Tyr(Bn) from the P_1 site of the inhibitors. While methane- and ethanesulfonamides were preferred over benzenesulfonamide, 2-thienylsulfonamide was equipotent to methanesulfonamide. However, an *N*-methyl methanesulfonamide was approximately 46 times less potent than the unsubstituted sulfonamide, suggesting that the NH of the sulfonamide moiety might be involved in energetically beneficial binding. Conversely, it is also possible that *N*-methyl methanesulfonamide assumes a different conformation than the preferred bioactive conformation offered by the parent methanesulfonamide.

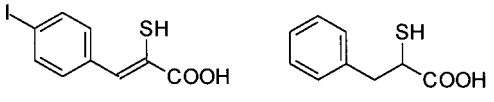
In extending the series, the authors also varied P_2-D-amino acids. While aromatic ring containing D-amino acids were all well tolerated, incorporation of D-Leu at P_2 resulted in loss of potency. This supported the original hypothesis that the presence of an aromatic moiety in the P_3 region is beneficial. Finally, incorporation of L-Ser(Bn) at P_2 resulted in a greater than 5-fold decrease in potency, revealing the importance of the D configuration at P_2 in this series of compounds. These novel compounds are active site-directed inhibitors. Two members of the series, methanesulfonyl-D-Ser(Bn)-Tyr(Bn)-H and methanesulfonyl-D-Phe-Phe-H were 11 times more selective for rh calpain I over cathepsin B. A number of compounds that displayed potent inhibitory activity in the enzyme assay also inhibited calpain I in human leukemic T cell line (IC_{50} = 0.3-0.8 μM). This study revealed 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 inhibitior; an *N*-alkyl- or arylsulfonyl-D-amino acid at P_2 can bind with high affinity.

Nonpeptidic inhibitors

Mercaptoacrylic acid derivatives

In order to develop selective and nonpeptidic calpain I inhibitors, Wang *et al.* screened more than 150,000 compounds. This led to identification of a series of mercaptoacrylic acid derivatives as potential calpain I inhibitors. A directed synthetic effort led to a series of potent inhibitors of which compound **18** (PD-150606) is a representative member (Fig. 9) (47, 48).

Compound **18** inhibited calpain I with a K_i of 0.21 μM and cathepsin B with a K_i of 127.8 μM. This compound was a nonactive site-directed inhibitor. At micromolar concentration, **18** inhibited calpain activity in two intact cell



18 **19**

Fig. 9.

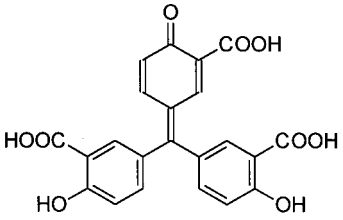


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Fig. 10

systems (human leukemic Molt-4 cells and human neuroblastoma cell line SY5Y). In a similar way, compound **18** (10 μM) made cerebral glutamatergic neurons more resistant to hypoxic/hypoglycemic challenge. This compound (100 μM) also protected cerebellar Purkinje neurons from AMPA (30 μM) toxicity. Interestingly, the corresponding reduced analog, compound **19** (PD-145305) was devoid of any activity. It was also shown that unmodified sulfhydryl and carboxylic acid groups were necessary for calpain I inhibition. The x-ray crystal structure of the Ca^{2+} bound domain VI of calpain complexed with compound **18** has been reported (49). The authors, however, cautioned that further improvements of the chemical properties of this class of mercaptoacrylic acid derivatives are needed before they can be useful in *in vivo* studies (48).

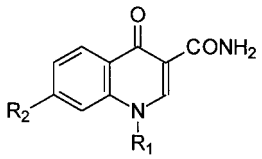
Aurintricarboxylic acid

Posner *et al.* reported aurintricarboxylic acid (ATA) to be a reversible inhibitor of calpain I (IC_{50} = 22 μM) (50). However, it should be noted that although the structure of commercially available ATA is as depicted (**20**, Fig. 10), it mainly exists as a complex heterogeneous mixture of polymers (51). Thus, the true identity of the active form(s) is debatable. It was noted that aurin, an ATA analog without the carboxyl groups, was inactive against calpain I. ATA is also an inhibitor of cathepsin B (IC_{50} = 6.3 μM).

Posner *et al.* (50) reported that in a fetal cerebrocortical culture model of excitotoxicity, pre- and posttreatment with ATA reduced *N*-methyl-D-aspartate (NMDA)-induced neuronal death, while application of ATA concurrent to NMDA challenge alone had no effect. They suggested that this pattern of protection could not be explained by simple NMDA receptor antagonism. They proposed that the neuroprotective effect of ATA could be in part due to its ability to inhibit calpain. ATA was previously shown to protect hippocampal neurons from glutamate excitotoxicity (52).

1,4-Dihydro-4-oxo-3-quinolinecarboxamides

Graybill *et al.* reported a series of 1,4-dihydro-4-oxo-3-quinolinecarboxamides to be nonpeptide inhibitors of human erythrocyte calpain I (53). An in-house screening



21) R_1 = 2'-CH_3-4'-HOC_6H_3, R_2 = 4"-pyridynil

22) R_1 = 2'-Cl-4'-HOC_6H_3, R_2 = 4"-pyridynil

23) R_1 = 2'-Cl-4'-HOC_6H_3, R_2 = 2"-pyrazolyl

Fig. 11.

effort produced a 3-quinolinecarboxamide as a potential lead. A high-throughput screening (>500 quinoline analogs) revealed that 1-(4'-hydroxyphenyl) moiety was essential for inhibition. Synthetic efforts then generated compounds **21-23** as the most potent (IC_{50}s = 0.5-0.6 μM) members of the series (Fig. 11).

SAR studies revealed that the carboxamide moiety is essential for potency. The authors invoked a planar, intramolecular H-bonded conformation of the β-keto amide functionality as the bioactive conformation. The compounds were reported to be reversible inhibitors of calpain I. However, they did not inhibit the enzyme by a competitive mechanism of action, indicating nonactive site-directed inhibition. Compound **22** was reported to be 50- and 44-fold more selective for human erythrocyte calpain I than cathepsin B and cathepsin L, respectively.

Miscellaneous

There have been reports in the literature about various other classes of calpain I inhibitors. For example, Foreman *et al.* reported an isocoumarin derivative to be a low affinity inhibitor of calpain I (IC_{50} = 10 μM) (54); however, the compound potently inhibits serine proteases also. On the other hand, Giordano *et al.* disclosed halohydrazides to be inhibitors of calpain (55). Alvarez and coworkers reported that a diketopiperazine of *N*-methyltyrosine and the tetrapeptide *N*-methyltyrosyl-*N*-methyltyrosyl-leucyl-alanine inhibited calpain in micromolar range (56). These compounds were isolated from an actinomycete strain *Streptomyces griseus.* Palmer *et al.* disclosed peptidyl vinyl sulfones to be inactivators of calpain I (57). There are also reports of novel structures as calpain I inhibitors in the patent literature which are beyond the scope of this review.

Conclusions

In recent years, the role of calpain I in various neurodegenerative disorders has become apparent. Thus, the discovery of potent inhibitors of calpain I remains an active area of research. With new generations of peptidomimetic and nonpeptidic inhibitors in hand, the exploration of the role of calpain I in various disease pathogenesis should be facilitated. These compounds should also act as guides for next generation inhibitors, ultimately leading to clinically useful drugs for various neurodegenerative disorders.

Acknowledgements

This review is dedicated to Mrs. Shelley Chatterjee for her courage and fighting spirit. The author expresses his sincere gratitude to Drs. Jeffry L. Vaught, James C. Kauer and John P. Mallamo for their continuing encouragement and support. Thanks are due to Ms. Beverly Cantor for her help with literature searching. The author is also grateful to the members of the discovery group of Cephalon, Inc. for their contributions and camaraderie.

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