Cysteine proteases as therapeutic targets: does selectivity matter? A systematic review of calpain and cathepsin inhibitors

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Abstract
Cysteine proteases continue to provide validated targets for treatment of human diseases. In neurodegenerative disorders, multiple cysteine proteases provide targets for enzyme inhibitors, notably caspasas, calpains, and cathepsins. The reactive, active-site cysteine provides specificity for many inhibitor designs over other families of proteases, such as aspartate and serine; however, a) inhibitor strategies often use covalent enzyme modification, and b) obtaining selectivity within families of cysteine proteases and their isozymes is problematic. This review provides a general update on strategies for cysteine protease inhibitor design and a focus on cathepsin B and calpain 1 as drug targets for neurodegenerative disorders; the latter focus providing an interesting query for the contemporary assumptions that irreversible, covalent protein modification and low selectivity are anathema to therapeutic safety and efficacy.

Abbreviations: AD, Alzheimer's disease; Ala, alanine; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; APP/PS1, Aβ overexpressing mice APP(K670N/M671L) and PS1(M146L) mutants; AppLon, London familial amyloid precursor protein mutation, APP(V717I); AppSwe, Swedish amyloid precursor protein mutation, APP(K670N/M671L); Arg, arginine; Aβ, amyloid β; Aβ1–42, amyloid β, 42 amino acid protein; BACE-1, β-amyloid cleaving enzyme; BBB, blood–brain barrier; CaMKII, Ca2+/calmodulin-dependent protein kinases II; CANP, calcium-activated neutral protease; Cdk5/p35, activator of cyclin-dependent kinase 5; CNS, central nervous system; CREB, cyclic adenosine monophosphate response element binding protein; DTT, dithioerythritol; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase 1/2; Gln, glutamine; Glu, glutamic acid; Gly, glutamine; GSH, glutathione; Hsp70.1, heat shock protein 70.1; Ile, isoleucine; isoAsp, isoaspartate; KO, knockout; Leu, leucine; Lys, lysine; MAP-2, microtubule-associated protein 2; Met, methionine; MMP-9, matrix metalloproteinase 9; NFT, neurofibrillary tangles; Nle, norleucine; PD, Parkinson's disease; pGlu, pyroglutamate; Phe, phenylalanine; PK, pharmacokinetic; PKC, protein kinase C; Pro, proline; PTP1B, protein-tyrosine phosphatase 1B; pyroGluAβ, pyroglutamate-amyloid β; SP, senile plaques; TBI, traumatic brain injury; Thr, threonine; TNF, tumor necrosis factor; Tyr, tyrosine; Val, valine; WRX, Trp-Arg containing epoxysuccinate cysteine protease inhibitor; WT, wildtype

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1. Introduction

Proteases are enzymes that irreversibly hydrolyze a peptide bond in an amino acid sequence by nucleophilic attack and subsequent hydrolysis of a tetrahedral intermediate. Proteases are grouped according to the key catalytic group in the active site: serine (Ser), threonine (Thr), cysteine (Cys), aspartate (Asp), glutamate (Glu), or zinc in metalloproteases. Ser, Cys and Thr act directly as nucleophiles that attack an amide carbonyl C, whereas Asp, Glu and metalloproteases activate a water molecule that then acts as a nucleophile. The enzymes are also classified into exopeptidases and endopeptidases by the position of the peptide bond in a protein they cleave. Exopeptidases truncate one or several amino acids from either the N- or the C-terminus of a peptide, whereas endopeptidases cleave an internal peptide bond. The catalytic site of CA-clan papain-like cysteine proteases consists of Cys, histidine (His) and Asp residues and is highly conserved among members of the enzyme family. This review will focus on approaches to inhibition of two families of protease enzymes, calpains and cathepsins, of interest in neurodegeneration and cancer therapy and the quixotic pursuit of selectivity.

2. Cysteine proteases

2.1. Cathepsins

Cathepsin inhibitors have been reviewed recently by Turk et al. and earlier by Hernandez and Roush. A review specific to cathepin B inhibitors has also been published by Frlan and Gobec. Cathepsins are a group of protease enzymes originally discovered in the cell lysosome, with several members ubiquitous in the human body. They are not catalytically conserved: cathepsins A, G are serine proteases; cathepsins D, E are aspartate proteases; and the remainder are lysosomal cysteine proteases, including the human isoforms B, C, F, H, K, L, O, S, V, X and W. Cathepsins B, F, H and L occur throughout the CNS, while C, S, V and X are expressed in specific cell types within the CNS. The pHmax for optimum cathepsin activity is slightly acidic, corresponding to the environment found in the lysosome. Although they have been traditionally viewed as enzymes involved in terminal protein degradation, knockout (KO) mice have revealed major roles in cell regulation, i.e. of cell proliferation and adhesion, apoptosis, lipid metabolism and immune response.

The crystal structure of a number of cathepsins has been determined, among them cathepsin B. Cathepsin B is unique among the cathepsins in that it has an occluding loop, a peptide sequence which when closed can hinder access to the primed side of the substrate pocket. Thus cathepsin B can function as an endo- or exopeptidase depending on pH. The occluding loop has been targeted for design of non-electrophilic cathepsin B inhibitors. The lysosomal cathepsin K occurs in osteoclasts and is a major factor in bone resorption and a target for treating osteoporosis. Several inhibitors are in development, with one, odanacatib, having reached phase III clinical trials. Table 1 shows residue preference of cathepsin B in peptide substrates in each position. Fig. 1 shows primed and unprimed amino acid residues in protease substrates and inhibitors.

2.2. Calpains

Calpains are neutral, cytosolic cysteine proteases with 15 isoforms reported, of which 11 have been identified in humans. The first reports characterizing members of the enzyme family emerged in 1964, naming the enzyme calcium-activated neutral protease (CANP).

| Table 1 | Cathepsin B: residue preference in peptide substrates in each position |
|---------|-------------------------------------------------|
| Unprimed Preference | Primed Preference |
| P1 | Gly > Ala, Met, Gin | P1’ | Phe > Gly |
| P2 | Val > Phe, Tyr | P2’ | Val, Ile > Gly, Thr |
| P3 | Gly > Lys, Phe | P3’ | Gly |

*aSee Fig. 1 for depiction of primed and unprimed sites. Ala, alanine; Gin, glutamine; Gly, glutamic acid; Ile, isoleucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Tyr, tyrosine; Val, valine.*

The enzymes consist of a catalytic subunit (82 or 80 kDa for calpains 1 and 2, respectively) and a Ca²⁺ binding subunit (28 kDa). The enzymes are unique among cysteine proteases in that the cystolic proenzyme is activated by Ca²⁺ ions, inducing a conformational change. This change drives spatial proximity of the catalytic triad to the regulatory subunit, domain I, and subsequent autocatalytic cleavage. The two most widely researched isoforms of calpain are ubiquitous, these are termed calpains 1 and 2, or μ- and m-calpain, requiring 5–30 μmol/L or millimolar Ca²⁺ for activation, respectively.

The presence of phospholipids or phosphoinositides can decrease the Ca²⁺ concentration required for the activation of calpain. The expression of calpains 1 and 2 can vary greatly depending on cell types and conditions. Other members of the calpain family are tissue-specific. The active sites and substrates of calpains 1 and 2 are very similar, and specific inhibitors have not been developed.

Calpains and cathepsins regulate the activity of other biomolecules through limited proteolytic cleavage at specific sites. The products of these enzyme catalyzed reactions are often functional proteins and therefore these cysteine proteases constitute important regulatory enzymes. Protease activation is a necessary cog in the
cellular machine under physiological conditions. Constant over-activation of calpain and other proteolytic enzymes, however, causes excessive protein degradation and neuronal death. Calpains play a key role in enzyme activation, platelet activation, cell proliferation and signal transduction.

Calpains have numerous protein substrates including G-proteins and cytoskeletal proteins such as spectrin, integrin and MAP-2. Calpain substrates that are protein kinases and further regulate the function and breakdown of cytoskeletal proteins are PKC, ERK1/2, CaMKII, Cdk-5/p35, Bid, and Bax. Transcription factors (c-Jun, c-Fos) and membrane receptors, e.g., EGFR, are also substrates of calpain. Calpain regulates the activity of a number of proteins that are part of processes influencing neuronal plasticity, cognition and neurodegeneration. CREB is a key protein in synaptic plasticity, impaired activation of which is a key contributor to pathogenesis of Alzheimer’s disease (AD) and Parkinson’s disease. CREB is a substrate of calpain, and thus inhibitors have been demonstrated to increase CREB phosphorylation, in turn restoring synaptic plasticity in the APP/PS1 transgenic mouse model of AD. KO of calpain 1 in mice has been shown to influence degradation of erythrocyte membrane proteins and platelet aggregation, reportedly via action on the calpain substrate PTP1B.

The peptide sequence of the endogenous, specific inhibitor, calpastatin, is known and the inhibition mechanism has been elucidated. Calpastatin binds to both P and P’ sides of the active site, but does not occupy the active site, thus avoiding self-immolation. Calpastatin regulates the proteolytic activity of calpains. Calpastatin is specific for the catalytically active form of calpain, bound to Ca2+, and consists of an N-terminal domain and four repeats of an inhibitory domain. The peptide sequence of the endogenous inhibitor has been truncated to generate calpain inhibitors. Improved cell permeability has been attempted by conjugation of appropriate peptide sequences (i.e., penetratin), but their clinical use in CNS indications is limited by the usual bioavailability challenges of oligopeptide drugs. Nevertheless, upregulation or decreased degradation of calpastatin is a therapeutic target in AD. Table 2 shows residue preference of calpain in peptide substrates at each position.

Abnormal activation or dysregulation of calpains has been linked to a number of pathological conditions. The increased intracellular Ca2+ levels in traumatic brain injury (TBI) and cerebral ischemia lead to increased calpain activation and secondary injury due to the degradation of cell membrane components. Calpain 1 is a target in chronic neurodegeneration occurring in AD, Parkinson’s disease (PD), Huntington’s disease, multiple sclerosis, and amyotrophic lateral sclerosis (ALS). Calpain 10 and the gene encoding it have been linked to type 2 diabetes mellitus. A mutation causing loss of function of calpain 3 is believed to be responsible for limb-girdle muscular dystrophy.

Cysteine protease inhibitors belong to two general classes: the most widely explored inhibitors use an electrophile to modify the active cysteine covalently and a recognition motif for binding to the active site; allosteric inhibitors have also been reported.

3. Electrophilic warheads for Cys protease inhibitors

3.1. Irreversible covalent inhibitors

Electrophiles that alkylate, acylate, phosphorylate or sulfonate the active site cysteine irreversibly, include simple non-selective alkylating or acylating agents such as iodoacetate, N-ethylmaleimide, and diisopropyl fluorophosphate. Examples of electrophilic warheads used in selective and potent cysteine protease inhibitors include epoxysuccinates, vinylsulfones, allyl sulfones, vinyl sulfonates, diazomethyl ketones and fluoro- or chloromethyl ketones. The latter were developed in the 1960s as inhibitors of trypsin and chymotrypsin and react with both serine and cysteine proteases. Halomethyl ketones can alkylate active site His residues and the activated ketone has been proposed to form a transition state analog at the active site. Moderation of the reactivity of halomethyl ketones led to the development of acylxymethyl ketones and other activated ketones such as arylxymethyl, sulfonium methyl and ketoheterocycles. Other examples are vinyl ketones, vinyl esters and vinyl sulfones, which provide alternate Michael acceptor electrophiles. Diazomethylketones have been explored and selectivity among different cathepsins attempted by the use of an appropriate recognition group.

3.1.1. Epoxysuccinates

Epoxysuccinates occupy an important role as Cys protease inhibitors since the discovery of E-64 (1, Fig. 2) from Aspergillus japonicus in 1977. Total synthesis of E-64 soon followed. A less hydrophilic derivative, E-64c (2), was designed later, targeted against muscular dystrophy, and its ethyl ester prodrug,
E-64d (2'), developed to overcome the poor absorption of E-64c, progressed to phase III clinical trials\cite{20,55}. The epoxide irreversibly modifies the active site Cys, forming a thioether bond\cite{20,28}. Epoxysuccinates are selective towards Cys proteases due to the nucleophilicity of the active site cysteine. Peptidomimetic recognition groups are used to increase binding, selectivity, and potency. The amino acid preference of calpains has been investigated by the generation of positional scanning epoxide libraries by Cuerrier et al.\cite{69} The studies showed that for inhibition of calpains 1 and 2, the preferred residues in the P3 and P4 positions are Trp and Arg (Table 1) This led to the development of the WRX series of calpain inhibitors (e.g. 6, Fig. 2). Members of this compound library were reported to have 3 to 6-fold selectivity towards calpain 2 vs. calpain 1 and significant selectivity for calpains over cathepsins. However, changing the Leu or Val at the P2 position to Tyr switched the selectivity towards cathepsin B\cite{70}. Calpain inhibitor reviews have appeared\cite{71,72,73,74,75,76}.

The chemical space around the P' substituent of epoxide-containing peptidomimetics has been explored by Meara et al.\cite{77,78}. Carboxylic acid derivatives of E-64c were synthesized. The potency for inhibition of papain and cathepsin B was reported to increase by orders of magnitude in the following ranking of epoxide P' substituents: \( \text{CH}_2\text{OH} < \text{COCH}_3 < \text{COOR} < \text{CONHOH} < \text{CONH}_2 \) (Fig. 2). Assay of calpain inhibition by a series of ester and amide derivatives of E-64c in intact and lysed platelets revealed that a number of haloethyl esters were comparable in cell permeability and stability to E-64d, while amides of epoxysuccinic acids seemed to be low-potency inhibitors. E-64c itself had too poor cell permeability to inhibit calpain in intact platelets. Other amide derivatives of E-64c that extended into the P' site were weak calpain inhibitors compared to the free acid\cite{76}.

The first highly selective inhibitor of cathepsin B, CA-074 (4, Fig. 2) was reported to exploit the exopeptidase activity of cathepsin B, unique among the other members of the cathepsins. CA-074 and its analogs bind to the occluding loop at the P' site. Its inactive methyl ester CA-074Me was designed to overcome poor cell permeability of the parent compound. CA-074 and CA-074Me were reported to undergo a loss of selectivity towards cathepsin B in the presence of GSH or dihydroerythritol (DTT)\cite{75,76}. The selectivity and bioavailability of epoxysuccinates was improved by substituting heterocyclic analogs for His at the P2 recognition group position by Schiefer et al.\cite{77}, resulting in the preclinical epoxysuccinate NYC-438 that reversed cognition deficits in the APP/PS1 AD mouse model and was devoid of toxicity even at 200 mg/kg.

### 3.1.2. Miscellaneous oxiranes and strained ring electrophiles

An arylsulfonyloxirane warhead was developed in 2013 as a cathepsin B, but the lack of a recognition group led to modest inhibition\cite{79}. Cyclic sulfates have been developed that show selectivity for cathepsin B over calpain, presumably due to the steric hindrance in the calpain active site\cite{80,81}. Other Cys protease inhibitors containing oxiranes, thiranes and aziridines were reviewed by Schirmeister et al.\cite{82} Vicik et al.\cite{83} explored a number of nitrogen-containing heterocycles in the P1 site of peptidomimetic cysteine protease inhibitors. Most compounds were micromolar inhibitors of cathepsin L, with selectivity against cathepsin B: the most potent compound had two electrophilic aziridines, with cathepsin L \( K_i = 13 \text{nmol/L} \) and cathepsin B \( K_i = 9.4 \mu\text{mol/L} \) (13, Fig. 3). This molecule conspicuously had an activity towards cathepsin L that exceeded that of all other inhibitors, hinting at a different binding mode.

Aziridines are inherently much more reactive to opportunistic biological nucleophiles than oxiranes; however, incorporation of N in an amide functionality attenuates this electrophilic reactivity. Miraziridine A (14, Fig. 3) is a natural product from a marine sponge, *Theonella aff. mirabilis*, with a reported cathepsin B IC\(_{50}\) of 2.1 \(\mu\text{mol/L} \)\cite{85}, and both a reactive aziridine and less reactive Michael acceptor \( \alpha,\beta\)-unsaturated carboxylate as terminal electrophiles.

\( \beta\)-Lactone and \( \beta\)-lactam electrophiles have been reported to acylate Ser and Thr residues at the active site of bacterial transpeptidases and have been used as antibiotics since the discovery of penicillin. With the appropriate recognition group, these four-membered rings (15, Fig. 3) also react with thiols in a Cys protease active site\cite{86}. A series of 6-substituted oxepanones was developed with the more potent inhibitor having 4 mmol/L potency against cathepsin L and good selectivity versus cathepsin B\cite{87}.

### 3.1.3. Michael acceptors

While fumaric acid derivatives were reported not to inhibit calpain and cathepsin B, similar Michael acceptors with an azapeptide recognition group (16, Fig. 4) have reported good activity against caspases\cite{88}. Adducts of caspases with these Michael acceptors formed by 1,4-conjugate addition have been identified by X-ray
crystallography. The fumaric acid derivative of E-64c (DC-11, Fig. 4) is 1000-fold less potent as an inhibitor of calpain 1 than E-64c, and is a weak irreversible inhibitor of cathepsins B and L. A related, potent inhibitor of falcipain 2 (K_i = 17 nmol/L, 18, Fig. 4) was developed as an antimalarial drug with good selectivity against cathepsins B and L (7.3 and 8.4 μmol/L).

Vinyl sulfone containing peptidomimetics with varied P2 amino acids were explored as inhibitors of cathepsins K, L and S. The inhibitors had highest potency for cathepsin S, reaching 13 nmol/L. Acylloxymethyl ketones had highest potency for cathepsin S, reaching 13 nmol/L. Acyloxymethyl ketones (Z-Phe-Ala-CH₂OCO-R) was inversely correlated with the pKₐ of the carboxylate leaving group, with the 2,6-bis-trifluoromethylbenzoate (24, Fig. 5) having the highest potency. The compounds also show potent inhibition of cathepsin L and S but not calpain 1. An alternative approach reported a sulfanylmethyl ketone (Z-Leu-Leu-Phe-CH₂S) (25, Fig. 5) that was a potent inhibitor of cruzain with good selectivity (cruazain K_i ~ 0.9 nmol/L, cathepsin B ~ 700 nmol/L, cathepsin L ~ 28.8 nmol/L). Unlike acyloxymethyl ketones, sulfonium methyl ketone peptidomimetics, e.g., Z-Leu-Leu-Phe-CH₂S(PhCH₃) (26, Fig. 5), inhibited calpain 1 with high potency.

3.2. Reversible inhibitors

Reversible Cys protease inhibitors are compounds forming a non-covalent complex with the enzyme. These inhibitors can bind to the active site without substrate bound (transition state analogs, competitive inhibitors) or with substrate already bound (uncompetitive inhibitors). A third type of reversible inhibitor binds to an allosteric site (non-competitive inhibitors). Reversible inhibitors do not form covalent adducts with the enzyme and can be removed by dialysis if the non-covalent binding affinity is not too high.

3.2.1. Aldehydes and ketones

Figure 5 Structures of diazomethyl, acyloxoy and other ketone cysteine protease inhibitors.

Figure 6 Structures of aldehyde and cyclopropenone inhibitors.

Activated ketones provide a leaving group for displacement by active site thiol, presumably via a mechanism similar to that shown above for the diazomethyl ketones. The benzotriazol-1-yl leaving group has been successfully utilized to inhibit calpain potently; however, this activated ketone was unstable in aqueous solutions. Tetrafluoro-phenoxymethyl ketones (e.g. 27) have been developed as potent cruzain inhibitors.

The 1,2,4-thiadiazole based inhibitors do not easily fit into the classes of irreversible covalent enzyme inhibitors discussed above, but the use of this thiophilic warhead for inhibition of cathepsin B (28: K_i ~ 2 μmol/L) deserves mention, providing selectivity over non-cysteine protease families.
be obtained from kinetic analysis, which has not always been carried out in sufficient detail for definitive conclusions. The most well studied examples are peptidomimetic aldehydes and trifluoromethyl ketones \(^{39-41}\). Peptide aldehydes were isolated from Streptomyces strains and were found to inhibit calpain and other proteases \(^{111}\). The physicochemical characteristics of the compounds were improved by substituting the terminal amino acid for a hydrophobic cap group such as benzylxycarbonyl, resulting in calpeptin (Z-Leu-Nle(norleucine)-H, 29 (Fig. 6), a 40 nmol/L inhibitor of human platelet calpain 2 \(^{113,114}\). Another cell permeable aldehyde inhibitor relying on the same principles is Z-Val-Phe-H (MDL 28,170, 30) \(^{118}\). When a phenylbutyryl group was substituted for Z, the resulting compound inhibited calpains 1 and 2 with potency of 36 and 50 nmol/L, respectively, but the compound showed some inhibition of trypsin, chymotrypsin and cathepsin H, demonstrating the lower selectivity of the aldehyde warhead \(^{110}\).

Using an acetyl cap group gave, Ac-Leu-Leu-Nle-H (31, Fig. 6) and Ac-Leu-Leu-Met-H (32, Fig. 6), which were named “calpain inhibitor I” and “calpain inhibitor II”, respectively. \(^{116}\) Calpain inhibitor I is a potent inhibitor of cathepsin L (0.5 nmol/L) and calpain inhibitor II, of cathepsin B (100 nmol/L). Peptidomimetic aldehydes can show high selectivity in biochemical assays; however, the chemical reactivity of aldehydes, leading to reversible Schiff base formation with proteins, metabolic oxidation and reduction, and pH-dependent hydrate formation, result in unsatisfactory stability and bioavailability, underlying a lack of progress to the clinic \(^{117}\). Nevertheless, these aldehydes are widely used as chemical probes for in vitro calpain inhibition.

Cyclic hemiacetals provide a prodrug approach to aldehyde inhibitors designed to increase the biological half-life and enhance PK properties. The aldehyde SJA-6017 (33, Fig. 6), IC\(_{50}\) = 0.022 μmol/L (calpain 1) and IC\(_{50}\) = 0.049 μmol/L (calpain 2), was found to prevent cataract formation in rats. The hemiacetal prodrg itself (34, Fig. 6) is less active: IC\(_{50}\) = 0.88 μmol/L (calpain 1), IC\(_{50}\) = 2.6 μmol/L (calpain 2) \(^{118}\).

### 3.2.2. Cyclopropenones

Cyclopropenones have been reported as reversible covalent inhibitors of calpain \(^{119}\). The major example is BDA-410 (35, Fig. 6) with reported IC\(_{50}\) of 130 nmol/L and 630 nmol/L for calpains 1 and 2, respectively. \(^{120}\) BDA-410 is orally bioavailable and has been reported to have a neuroprotective effect in AD mouse models \(^{121}\). Potential mechanisms of action include both 1,2- and 1,4- addition to the cyclopropenone ring and charge-transfer complex formation at the active site involving protonation of the cyclopropenone to a stabilized aromatic hydroxycyclopropenyl cation \(^{122}\).

### 3.2.3. α-Keto derivatives

A carbonyl group adjacent to an acyl group, usually a carboxylate ester/amide, or a heterocycle, provides an electrophile for reversible addition of a nucleophile Cys, Ser, or Thr at an enzyme active site. α-Ketoacids, α-ketoesters, and α-ketoamides are not transition state analogs, but have the ability to form a tetrahedral transition state analog on addition of the enzyme nucleophile at the active site. Substitution of the aldehyde moiety of known calpain inhibitors with α-keto warheads has been a popular approach. In α-ketoacids, H-bonding with an active site His has also been proposed to contribute to inhibition to rationalize more potent inhibition compared to esters or amides: Z-Leu-Phe-COOH (36, Fig. 7) (calpain 1 K\(_i\) = 8.7 nmol/L, calpain 2 K\(_i\) = 5.7 nmol/L) \(^{123}\). The P\(_1\) and P\(_2\) peptide residues and hydrophobic cap group were explored extensively. In the α-ketoester series, selectivity up to 12-fold towards calpain 2 vs. calpain 1 was reported, with the best inhibitor (37, Fig. 7) providing calpain 1 K\(_i\) = 100 nmol/L and calpain 2 K\(_i\) = 200 nmol/L. This performance was bettered by the corresponding N-monosubstituted α-ketoamides: 20 \(< K_i < 200 \text{ nmol/L for calpains 1 and 2 and micromolar inhibition of cathepsin B. Calpain inhibitor, AK295 (38, Fig. 7), was reported to have neuroprotective effects in a rat model of cerebral focal ischemia.}^{24}\)

The chemical space around the P3 cap group of ketoamides was extensively explored, resulting in the creation of A-705239 (39) and A-705253 (40, Fig. 7), non-selective inhibitors of calpain (13.3 nmol/L, 27 nmol/L) and cathepsin B (27 nmol/L, 62 nmol/L), but with improved water solubility, cell permeability, and metabolic stability. \(^{111}\) A-705239 rescued brain cells in a model of fluid percussive traumatic brain injury \(^{111}\). Inhibitors with a heterocyclic cap group, such as quinoline carboximides and chromene derivatives were described, along with ring-opened 4-aryl-4-oxobutanolic acid derivatives \(^{125,126}\). These calpain inhibitors have been developed by the cyclization and conformational restriction of the P3 amide and recognition group of conventional ketoamide inhibitors. Ketoamide inhibitors based on the structure of A-705239 and its analogs have been further developed by Abbvie \(^{127}\). The modifications included bioisoster substitutions of the diarylalkene by substituted and fused pyrazoles and replacement of the central benzene ring with pyridine and oxopyrrole \(^{128-131}\). ABT-957, currently in clinical trials for AD, represents the culmination of the ketoamide approach to calpain inhibition in neurodegenerative disorders. Two representative chemical structures published by Abbvie are shown \((41, 42, Fig. 7)\).

α-Ketoheterocycles have been widely explored as inhibitors of many non-cysteine and cysteine proteases, with a number of examples of cathepsin inhibitors previously reviewed \(^{32}\). Early examples, were developed by optimization of P\(_1\), P\(_2\), and P3 interactions \(^{133}\). Cathepsin K inhibitors were described with K\(_i\) = 1.7–54 nmol/L and reported selectivity over cathepsins B and
L (e.g. 43: cathepsin K $K_i = 1.7$ nmol/L, cathepsin S $K_i = 350$ nmol/L, cathepsin L $K_i = 220$ nmol/L, cathepsin B $K_i = 1$ μmol/L; Fig. 7). Other examples showed high potency, but low selectivity: $IC_{50} = 0.25–1$ nmol/L for cathepsins B, L, K and S.134 Similar compounds with variations in the P2–P3 recognition motif were reported as inhibitors of cathepsin S, some of them (e.g. 44, Fig. 7): achieving a 100-fold selectivity vs. cathepsins B, K and L.135

Cyclic ketone inhibitors of cathepsin K have been described that have been developed by locking alkoxymethyl or alkylaminomethyl ketones into an aza- or oxacycle to induce conformational restraint. Such compounds are reported as noncompetitive reversible inhibitors of cathepsin K.136 SB-357114 (45, Fig. 7) was reported to inhibit human cathepsin K with a $K_i$ of 0.16 nmol/mL, and cathepsins L, S and B with $K_i$ values of 2.2, 4.0, and 500 nmol/L, respectively, yielding a marked reduction in bone resorption in a nonhuman primate model of postmenopausal bone loss.137

3.2.4. Nitriles

Nitrile warheads have been traditionally targeted at cysteine proteases, although the increased reactivity of the related carbodiimide warhead has also been pursued for serine proteases. Several examples of nitriles inhibiting cathepsin K have been reported.138 As introduced above, odanacatib (46, Fig. 8) has progressed to clinical trials for osteoporosis. Drug optimization incorporated an N-(1-cyanocyclopropyl)acetamide warhead to reduce the metabolic lability of less substituted nitrile warheads. Monoalkylated acetamides have been developed as potent inhibitors of cathepsin S (the most potent example is 47, $K_i = 15$ nmol/L; see Fig. 8) with $>1000$-fold selectivity over cathepsins B, K and L.

Selective cathepsin L inhibitors containing substituted and unsubstituted cyanomethylene warheads have been reported, the most potent (48, Fig. 8) having an $IC_{50}$ of 1.26 nmol/L.139,140 The reactivity of carbodiimides towards nucleophilic addition is greater than that of nitriles. Examples of these, N-cyanopyrrolidines (Fig. 8, e.g. 49, $IC_{50}$ cathepsin L 50 nmol/L, K 80 nmol/L, B 1.4 μmol/L) and N-cyanoazetidines (e.g. 50, $IC_{50}$ cathepsin L 5 nmol/L, K 6 nmol/L, B 150 nmol/L), have been prepared as reversible inhibitors of cathepsins K, L, and B; expected to form a thiourea intermediate at the active site. The higher potency of the azetidine derivatives was attributed to the higher electrophilic reactivity. The formation of an isothiourea with the active site cysteine has been detected.141 2-Cyanopyrrolidines were described as selective cathepsin L inhibitors, the most potent compound (51, $K_i = 5.3$ μmol/L, Fig. 8) was reported to be selective against cathepsin B.142

The 1,3,5-triazine-2-carbonitrile represents another approach to modulating the reactivity of the nitrile warhead, including potent inhibitors such as 52 (Fig. 8; $K_i = 9$ nmol/L, rhodasin; 2 nmol/L, cathepsin L).143 Similarly, Merck have reported selective cathepsin S inhibiting purine-6-carbonitriles (e.g. 53, Fig. 8) that had been designed to exploit the differences between the active sites of cathepsins K and S, but aqueous stability was too poor for clinical progress.144 Merck has reported other N-heterocycles as cathepsin inhibitors, such as 6-phenyl-1H-imidazo[4,5-c]pyridine-4-carbonitriles (54, Fig. 8: $IC_{50}$ cathepsin S 6.9 nmol/L, cathepsin K 117 nmol/L).145 AstraZeneca has optimized pharmacokinetic properties of aminoacetanilide inhibitors of cathepsin C working towards a clinical candidate, reporting an $IC_{50}$ of 1 nmol/L and excellent selectivity for 55 (Fig. 8).146

4. Non-covalent inhibitors

The endogenous inhibitor of calpain, calpastatin, interacts with both the unprimed and primed sites of the enzyme without extending into the active site. Truncated peptidic inhibitors were designed based on the structure. Although this review is focused on covalent inhibitors of cysteine proteases, examples of allosteric inhibitors deserve brief mention. Early research identified mercaptoacrylates as cell-permeable, selective, noncompetitive inhibitors of calpain (i.e. PD-150606, 56 and PD-1517146, 57, Fig. 9), which were later reported to bind at an allosteric site on the Ca-binding domain VI.147,148 Neuroprotective activity was reported in models of ischemia and in electrophysiological studies.149 More recently, biphenyl-containing high potency calpain inhibitors were reported by Montero et al.150–152 (e.g. $IC_{50}$ 58, 98 nmol/L, and 59, 24 nmol/L; Fig. 9). Although the authors proposed the chelation
of Ca\(^{2+}\) as the mechanism of action, the variability of reported potency with the peptide sequence hints at an alternative allosteric mechanism. Macrocyclic compounds incorporating biphenyls were designed to improve physicochemical properties, at a significant cost in potency\(^{153}\).

5. Covalent vs. non-covalent inhibition

Irreversible covalent enzyme inhibitors react with the target enzyme after binding to it, and thus enzyme inactivation is not an equilibrium process as with reversible inhibition, and requires re-expression of the enzyme to reverse drug action, which may occur after elimination of the drug from the body. There has been a tendency to avoid irreversible, covalent inhibitors in drug development to avoid the risk of: 1) unpredictable side effects such as the generation of allergenic modified proteins (haptens); 2) non-specific, irreversible modification of off-target proteins; and 3) the difficulty in tracking metabolites when covalently bound to proteins. The increased toxicity of “covalent drugs” has been widely perceived, despite studies suggesting otherwise, such as the lack of correlation of thiol conjugate formation with the \textit{in vivo} toxicity of 50 approved drugs\(^{154}\). Aspirin is a textbook example of a covalent drug; however, it has taken the advent of covalent kinase inhibitors in cancer therapy to open the floodgates to such drugs\(^{155}\).

6. Therapeutic applications

6.1. Cathepsin inhibitors in cancer therapy

Inhibitors of cathepsins S, K, B and L have advanced to clinical trials in osteoporosis\(^{10}\) and cancer\(^{156}\). Cathepsins B and L are proposed biomarkers in cancer, with expression usually inversely correlated with outcome, for example, cathepsin B activity is increased in lung tumors and lymph node metastases\(^{157}\), and correlates with poor prognosis in metastatic non-small cell lung cancer\(^{157}\). Cathepsin B has been shown to have multiple roles in cancer, including tumor invasion, the formation of metastases and neovascularization, and is a pro-metastatic enzyme\(^{158}\). CA-074 inhibited the formation of bone metastases in breast cancer\(^{159}\); however, JPM-OEt, a prodrug of an inhibitor of cathepsins B and L, was ineffective in preventing metastases in breast cancer\(^{159}\). In a further mouse breast cancer model, CA-074 but not JPM-OEt, was found to decrease tumor invasion, neovascularization, and bone metastases\(^{160}\). The bioavailability of cathepsin inhibitor drug generated by the prodrug, JPM-OEt, is problematic\(^{161}\).

6.2. Neurodegenerative diseases

Calpains and cathepsins play key roles in TBI, ischemic brain injury, and in normal proteolytic and regulatory pathways in the brain involved in signaling and synaptic and neuronal plasticity\(^{162}\). Increased proteolytic activity is observed in neurodegenerative diseases and numerous studies have been conducted to elucidate the role of not only calpains and cathepsins, but also caspases\(^{163,164}\). Calpain 1 is highly expressed in neurons\(^{165}\) and calpain 2 in glial cells\(^{166}\). Elevated glutamate levels associated with excitotoxicity cause an influx of Ca\(^{2+}\) into neurons and consequent abnormal and extended hyperactivation of calpain. Chronic calpain activation, as opposed to transient activation, is associated with the breakdown of cell membranes, increased permeability of lysosomal membranes, and elevation of intracellular cathepsin levels\(^{160}\). Inhibition of both calpain and cathepsins has been reported to provide neuroprotection after cerebral ischemia\(^{169}\). A dose-dependent reduction in infarct volume by MDL 28,170, an aldehyde calpain inhibitor, was observed in a rat cerebral ischemia model\(^{169}\). Indeed, numerous studies have concluded that inhibition of cysteine proteases is neuroprotective in models of brain injury, and since these inhibitors often lack selectivity, the need for specific inhibitors needs examining\(^{91,169-172}\).

In TBI, secondary injury occurs after the initial insult as ion homeostasis is disturbed, excitatory mediators and reactive oxygen species are produced. As a consequence of cytosolic ion concentration change, calpains are activated and form part of a cascade of events leading to cell membrane breakdown, apoptotic and necrotic cell death\(^{167}\). The activity of cathepsin S was found to be increased in mice 2–4 h after TBI, indicating that cathepsin S is one of the enzymes causing secondary damage occurring after TBI. Neurological abnormalities were found to be decreased in mice that underwent TBI with prior intracerebral injection of LHVS, a vinyl sulfone cathepsin S inhibitor that does not penetrate the blood–brain barrier\(^{161}\). LHVS also inhibits other cathepsins with lower affinity\(^{90}\). In AD, cathepsin B is found throughout the brain and also in neurites and dendrites, whereas in normal brains cathepsin B activity is localized in lysosomes\(^{173,174}\). The localization of cystatins, the endogenous inhibitors of cathepsins, is also altered in neurodegenerative diseases\(^{173}\). Neurofibrillar tangles (NFTs) have been reported to contain increased amounts of calpain 2 and cathepsins\(^{175}\). Calpain 1 is known to be hyperactivated in brains of AD patients\(^{23}\). The level of calpastatin, the endogenous inhibitor of calpain, is also decreased\(^{176}\). A deuterated analog of E-64d, a pan-cysteine protease inhibitor known to inhibit calpains 1 and 2 as well as cathepsins B and L, is in clinical trials for AD therapy\(^{71,177,178}\).

6.3. The calpain–cathepsin hypothesis of neuronal loss

It is widely accepted that neuronal loss through neurodegeneration is a central event in the course of many acute and chronic disorders of the central nervous system such as cerebral ischemia, trauma and AD. The “calpain–cathepsin hypothesis” was formulated to provide a mechanism for neuronal death based upon experimental observations in the ischemic monkey paradigm. The hypothesis posits that calpain 1 hyperactivation compromises the lysosomal membranes and causes the release of cathepsins into the cytoplasm\(^{179}\). Calpain activation has been confirmed in the ischemic monkey brain\(^{180}\) and in brains of AD patients\(^{23,181}\). Recent data also suggests a dual role for Hsp70 as a chaperone for damaged proteins and as an important factor in the maintenance of lysosomal integrity. Calpain-mediated cleavage of Hsp 70.1 that has been modified by oxidative stress may impair lysosomal autophagy\(^{182,183}\).

Cathepsins released into the cytoplasm simultaneously damage the lysosomal membrane from outside and attack mitochondria, releasing cytochrome c and activating pro-apoptotic factors such as caspases-9 and caspase-3\(^{184,185}\). Several gene KO studies and pharmacological inhibitor experiments support this hypothesis. For example, the cathepsin B inhibitor, CA-074Me, reduced biomarkers of apoptosis, such as Bax, and neuronal cell death, and reduced memory loss in a TBI model\(^{186}\). Moreover, cathepsin B...
has been shown to be critical to TNF-α-mediated apoptosis by experiments in a KO mouse. Interestingly, pro-apoptotic activation was profoundly suppressed by cysteine protease inhibitors leupeptin and E64. However, CA-074, an epoxysuccinate cathepsin B inhibitor, did not inhibit digition-mediated caspase activation, indicating that not only lysosomal cathepsin B but also other lysosomal cysteine proteases are involved in the cascade leading to neuronal death. In line with this postulate, the abnormally high concentrations of cathepsins D and cathepsin L in the cytosol, can activate Bid through proteolysis and cause the release of cytochrome c from mitochondria as well as the activation of caspase-9 and caspase-3.

6.4. Are specific inhibitors essential for clinical success?

In TBI, a substantial increase in cathepsin B brain levels and activity correlated with neuronal cell death and behavioral dysfunction. E-64d treatment in a TBI mouse model led to similar improvements in WT compared to E-64d-treated cathepsin B KO mice, suggesting that E-64d, a non-selective cysteine protease inhibitor, functions primarily through cathepsin B inhibition in TBI. However, at one day post-trauma, E-64d-treated cathepsin B KO mice showed faster recovery of the motor functions than was observed for untreated cathepsin B KO mice, indicating a neuroprotective role for “off-target” inhibition of calpains, which are also validated drug targets in TBI. Indeed, brain calpain activity spikes within 24 h of trauma, and E-64d administration has been shown to reduce calpain activity and provide neuroprotection after trauma.

Therefore, in TBI treatment, some additional benefits of E-64d may occur through inhibition of both cathepsin B and calpain 1, although other targets cannot be excluded. The benefits of E-64d treatment in a focal ischemia animal model were attributed to inhibition of cathepsin B, calpain 1, and matrix metallopeptide-9 (MMP-9), a known contributor to TBI, although the mechanism of indirect inhibition of MMP-9 by E-64d is not known.

In AD, ischemic pathology was noted in the first description of disease neuropathology by Alois Alzheimer. Remarkably, the majority (~90%) of AD patients show a cerebral amyloid angiopathy that causes cerebral ischemia. It is therefore logical to propose that the calpain-cathepsin cascade, associated with ischemic neuronal death, contributes to AD pathogenesis. In AD brains, calpain 1 activity is increased 7-fold compared to age-matched brains. Amyloid precursor protein (APP) and amyloid β (Aβ) were also reported to induce calpain activation, and evidence exists for reciprocal processing by calpain of APP and tau proteins. Activated calpain was observed to occur in neurofibrillary tangles, senile plaques (SP), and dystrophic neuritis. Similarly, cathepsin D was observed to be localized extracellularly within senile plaques by immunooasays. In line with this observation, an age-dependent significant increase of cathepsin D levels and activity was documented in AD human brains suggesting a possible relationship between cathepsin D activation and SP formation.

In activated microglia, cathepsin B was claimed to be a key player in Aβ-induced neuronal death. Interestingly, this activated microglia-mediated neurotoxicity was corrected by cathepsin B gene knockdown as well as by the cathepsin B inhibitor CA-074. Accordingly, cathepsin B was proposed to mediate neuronal death initiated by inflammatory response to Aβ. Extra-lysosomal release of cathepsins has a major role in neuronal loss in AD. In this context, cathepsin B has been proposed to be an alternative executor of β-secretase activity, possessing excellent kinetic efficiency and specificity for cleaving wild-type APP at the β-secretase site in sporadic AD; cathepsin B may be key to amyloidogenesis in 99% of AD cases. E-64d treatment rescued memory function, and decreased brain Aβ1–28/Aβ1–42 and amyloid plaque neuropathology in AD animal models expressing human APP containing the wild-type β- and London mutant γ-secretase site (APPLon) sequences. Cathepsin B inhibition had no effect on Aβ pathology in mice expressing APP containing the Swedish mutant β-secretase site sequence (APPswe). Nevertheless, BDA-410 and E-64 improved memory deficits in APPswe mice in the absence of effects on Aβ, possibly by inhibition of calpain 1.

In contrast to cathepsin B, cathepsin D displays equivalent kinetic activity to BACE-1, cleaving the Swedish mutant β-secretase site more efficiently than the wild-type sequence. Importantly, relevant cathepsin D levels are about 280-fold greater than BACE-1. In the APPswe mutant, an asparagine residue replaces lysine in the wild-type protein. This P2 residue, is an important determinant of substrate specificity for proteases including cathepsins. Therefore, it is possible that several cysteine proteases are involved in processing the different APP mutations in familial AD. Cathepsin B, but not BACE-1, efficiently cleaves the wild-type β-secretase site containing isoaspartate (isoAsp) post-translational modification that is abundant in AD brains, leading to further N-terminal truncated and modified, neurotoxic Aβ peptide species such as pyroGluAβ. In turn, cathepsin B may be involved in the production of pGlu forms of Aβ that aggregate at accelerated rates.

Given evidence for multiple roles for calpain and cathepsins in neurodegeneration, both independent of and associated with hallmark AD pathology (Aβ and tau), it is difficult to conclude that therapy will be unsuccessful without an entirely selective inhibitor of one specific calpain or cathepsin isoform. Although calpain inhibitors, theoretically may be effective in very, early presymptomatic AD, diagnosis of this disease stage is not currently possible. Therefore, the pharmacological inhibition of both calpain and “later” mediators of neuronal death (cathepsins B, L, and D) would seem a reasonable approach supported by results in animal models with agents such as E-64, E-64d, and NYC-438.

References

1. Lecaille F, Kaleta J, Brömme D. Human and parasitic papain-like cysteine proteases: their role in physiology and pathology and recent developments in inhibitor design. Chem Rev 2002;102:4459–88.
2. Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, et al. Cysteine cathepsins: from structure, function and regulation to new frontiers. Biochim Biophys Acta 2012;1824:68–88.
3. Hernandez AA, Rouss WR. Recent advances in the synthesis, design and selection of cysteine protease inhibitors. Curr Opin Chem Biol 2002;6:459–65.
4. Gobec S, Frän R. Inhibitors of cathepsin B. Curr Med Chem 2006;13:2309–27.
5. Cheng XW, Huang Z, Kuzuya M, Okumura K, Murohara T. Cysteine protease cathepsins in atherosclerosis-based vascular disease and its complications. Hypertension 2011;58:978–86.
6. Turk V, Turk B, Turk D. Lysosomal cysteine proteases: facts and opportunities. EMBO J 2001;20:4629–33.
7. Musil D, Zucic D, Turk D, Engh RA, Mayr I, Huber R, et al. The refined 2.15 Å X-ray crystal structure of human liver cathepsin B: the structural basis for its specificity. EMBO J 1991;10:2321–30.
8. Illy C, Quraishi O, Wang J, Purisima E, Vernet T, Mort JS. Role of the occluding loop in cathepsin B activity. J Biol Chem 1997;272:1197–202.
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9. Schenker P, Alfaran P, Kolb P, Callisch A, Baici A. A double-headed cathepsin B inhibitor devoid of warhead. *Protein Sci* 2008;17:2145–55.

10. Bone HG, Dempster DW, Eismann JA, Greenspan SL, McClung MR, Nakamura T, et al. Odanacatib for the treatment of postmenopausal osteoporosis: development history and design and participant characteristics of LOFT, the long-term odanacatib fracture trial. *Osteoporos Int* 2015;26:699–712.

11. Cotrin SS, Puzer L, de Souza Judice WA, Juliano L, Carmona AK, Juliano MA. Positional-scanning combinatorial libraries of fluorescence resonance energy transfer peptides to define substrate specificity of carboxypeptidases: assays with human cathepsin B. *Anal Biochem* 2004;335:244–52.

12. Biniossek ML, Nügler DK, Becker-Pauly C, Schilling O. Proteomic identification of protease cleavage sites characterizes prime and non-prime specificity of cysteine cathepsins B, L, and S. *J Proteome Res* 2011;10:5363–73.

13. Huang YH, Wang KKW. The calpain family and human disease. *Trends Mol Med* 2001;7:355–62.

14. Sorimachi H, Hata S, Ono Y. Impact of genetic insights into calpain phosphorylase B kinase by Ca2+ fraction of rat brain. *J Biochem* 1983;94:149–55.

15. Meyer WL, Fischer EH, Krebs EG. Activation of skeletal muscle phosphorylase B kinase by Ca*-activated neutral protease in muscle. *Biochemistry* 1964;3:1033–9.

16. Meyer WL, Fischer EH, Krebs EG. Activation of skeletal muscle phosphorylase kinase by calcium ions. II. Identification of the kinase activating factor as a proteolytic enzyme. *Biochemistry* 1968;7:1126–16.

17. Goll DE, Thompson VF, Li HQ, Wei W, Cong JY. The calpain prime specificity of human cathepsins B, L, and S. *J Biol Chem* 2004;279:355–60.

18. Hirai S, Kawasaki H, Yaniv M, Suzuki K. Degradation of transcription factors, c-Jun and c-Fos, by calpain. *FEBS Lett* 1991;287:57–62.

19. Teich AF, Arancio O. Is the amyloid hypothesis of Alzheimer’s disease therapeutically relevant? *Biochem J* 2012;446:165–77.

20. Wang A, Bibb JA. Is CREB the angry bird that releases memory in Alzheimer’s? *Neuropharmacology* 2011;62:2145–49.

21. Saura CA, Valero J. The role of CREB signaling in Alzheimer’s disease and other cognitive disorders. *Rev Neurosci* 2011;22:153–69.

22. Pugazhenthi S, Wang MR, Pham S, Sze CJ, Eckman CB. Down-regulation of CREB expression in Alzheimer’s brain and in Aβ-treated rat hippocampal neurons. *Mol Neurodegener* 2011;6:60.

23. Satoh J, Tabanoki H, Arima K. Molecular network analysis suggests aberrant CREB-mediated gene regulation in the Alzheimer disease hippocampus. *Dis Markers* 2009;27:239–52.

24. Trinchese F, Fa MR, Liu SM, Zhang H, Hidalgo A, Schmidt SD, et al. Inhibition of calpains improves memory and synaptic transmission in a mouse model of Alzheimer disease. *J Clin Invest* 2008;118:2796–807.

25. Wieschhaus A, Khan A, Zaidi A, Rogalin H, Hanada T, Liu F, et al. Calpain-1 knockout reveals broad effects on erythrocYTE deformability and physiology. *Biochem J* 2012;448:141–52.

26. Kuchay SM, Kim N, Grunz EA, Fay WP, Chishti AH. Double knockouts reveal that protein tyrosine phosphatase 1B is a physiological target of calpain-1 in platelets. *Mol Cell Biol* 2007;27:6038–52.

27. Harris F, Biswas S, Singh J, Dennison S, Phoenix DA. Calpains and their multiple roles in diabetes mellitus. *Ann N Y Acad Sci* 2006;1084:452–80.

28. Sreenan SK, Zhou YP, Otani K, Hansen PA, Currie KP, Pan CY, et al. Calpains play a role in insulin secretion and action. *Diabetes* 2001;50:2013–20.
53. Richard I, Broux O, Allamand V, Fougerousse F, Chiannilkulchai N, Bourg N, et al. Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell 1995;81:27–40.

54. Fennell BD, Warren JM, Chung KK, Main HL, Arend AB, Tochowicz A, et al. Optimization of peptidyl allyl sulfoxones as clan CA cysteine protease inhibitors. J Enzyme Inhib Med Chem 2013;28:468–78.

55. Rasnick D. Synthesis of peptide fluoromethyl ketones and the inhibition of human cathepsin B. Anal Biochem 1985;140:461–5.

56. Rauber P, Angliker H, Walker B, Shaw E. The synthesis of peptidylfluoromethanes and their properties as inhibitors of serine proteinases and cysteine proteinases. Biochem J 1986;239:633–40.

57. Powers JC, Asgian JL, Ekiçi ÖD, James KE. Irreversible inhibitors of serine, cysteine, and threonine proteases. Chem Rev 2002;102:4639–750.

58. Ettari R, Micale N, Schirmeister T, Gelhaus C, Leippe M, Nizi E, et al. Novel peptidomimetics containing a vinyl ester moiety as highly potent and selective falcipain-2 inhibitors. J Med Chem 2009;52:2157–60.

59. Dunny E, Doherty W, Evans P, Malthouse JPG, Nolan D, Knox AJS. Vinyl sulfone-based peptidomimetics as anti-trypansomal agents: design, synthesis, biological and computational evaluation. J Med Chem 2013;56:6638–50.

60. Angliker H, Wikstrom P, Kirschke H, Shaw E. The inactivation of the cysteinyloxepidiazolines cathepsin H and C by affinity-labelling reagents. Biochem J 1989;262:63–8.

61. Crawford C, Mason RW, Wikstrom P, Shaw E. The design of peptidyl diazomethane inhibitors to distinguish between the cysteine proteinases calpain II, cathepsin L and cathepsin B. Biochem J 1998;335:751–8.

62. Shaw E, Mohanty S, Colic A, Stoka V, Turk V. The affinity-labelling of cathepsin S with peptidyl diazomethy ketones: comparison with the inhibition of cathepsin L and calpain. FEBS Lett 1993;334:340–2.

63. Hanada K, Tamai M, Yamagishi M, Ohnura S, Sawada J, Tanaka I. Isolation and characterization of E-64, a new thiol protease inhibitor. Agric Biol Chem 1978;42:523–8.

64. Hanada K, Tamai M, Ohnura S, Sawada J, Seki T, Tanaka I. Structure and synthesis of E-64, a new thiol protease inhibitor. Agric Biol Chem 1978;42:529–36.

65. Satoyoshi E. Therapeutic trials on progressive muscular dystrophy. Int Med 1992;31:841–6.

66. Tamai M, Matsumoto K, Omura S, Koyama I, Ozawa Y, Hanada K. In vitro and in vivo inhibition of cysteine proteinases by EST, a new analog of E-64. J Pharmacol Sci 1986;9:672–7.

67. Kerr ID, Lee JH, Pandey KC, Harrison A, Sajid M, Rosenthal PJ, et al. Structures of falcipain-2 and falcipain-3 bound to small epoxides. including cathepsins B, H and L. Biochem J 1992;282:1059–1069.

68. Hanada K, Tamai M, Yamagishi M, Ohnura S, Sawada J, Tanaka I. Isolation and characterization of E-64, a new thiol protease inhibitor. Agric Biol Chem 1978;42:523–8.

69. Molodecku T, Campbell RL, Cuerrier D, Davies PL. Crystal structures of calpain-E64 and -leupeptin inhibitor complexes reveal mobile loops gating the active site. J Mol Biol 2004;343:1313–26.

70. Cuerrier D, Molodecku T, Campbell RL, Kelly J, Youk B, Verhelst SHL, et al. Development of calpain-specific inactivators by screening of positional scanning epoxide libraries. J Biol Chem 2007;282:9680–110.

71. Giordano C, Calabretta R, Gallina C, Consalvi V, Scardura R, Noya FC, et al. Iodo and diiodotyrosine epoxysuccinyl derivatives as selective inhibitors of calpain B. Eur J Med Chem 1993:28:917–26.

72. Donkor JO. A survey of calpain inhibitors. Curr Med Chem 2000;7:171–9.

73. Donkor JO. An updated patent review of calpain inhibitors (2012–2014). Expert Opin Ther Pat 2015;25:17–31.

74. Pietsch M, Chua KCH, Abell AD. Calpains: attractive targets for the development of synthetic inhibitors. Curr Top Med Chem 2010;10:270–93.

75. Meara JP, Rich DH. Mechanistic studies on the inactivation of papain by epoxysuccinyl inhibitors. J Med Chem 1996;39:3537–66.

76. Biłohorski R, Powers JC, Kam CM, Walton R, Loewi RC. Further evidence for the importance of free carbamoyl in epoxysuccinate inhibitors of thiol proteases. J Enzyme Inhib Med Chem 1993;7:15–25.

77. Huang ZY, McGowan EB, Detwiler TC. Ester and amide derivatives of E64 as inhibitors of platelet calpains. J Med Chem 1992;35:2048–50.

78. Steverding D. The cathepsin B-selective inhibitors CA-074 and CA-074Me inactivate cathepsin L under reducing conditions. Open Enzyme Inhib J 2011;4:11–6.

79. Montaser M, Lalamnach G, Mach L, CA-074Me is a selective inhibitor of cathepsin B within living cells. Biol Chem 2002;383:1305–8.

80. Schiefer IT, Tapadar S, Litosh V, Siklos M, Scism R, Wijewickrama GT, et al. Design, synthesis, and optimization of novel peptides incorporating peptidomimetics as selective calpain inhibitors. J Med Chem 2013;56:6054–68.

81. Dana D, Davalos AR, De S, Rathod P, Gamage RK, Huestis J, et al. Development of cell-active non-peptidyl inhibitors of cysteine cathepsins. Bioorg Med Chem 2013;21:2975–87.

82. Hoge TR, Crawford KB. Enolate and other carbon nucleophile alkylation reactions using 1,2-cyclic sulfates as terminal epoxide equivalents. J Org Chem 1994;59:520–2.

83. Haruta J, Tanaka M. Uchida I, Ohtu A, Hara S, inventors; Japan Tobacco Inc, assignee. 1,3,2-dioxathioliane oxide derivative. European Patent Application EP 0460239; 1991 Dec 11.

84. Schirmeister T, Klockow A. Cysteine protease inhibitors containing small rings. Mini Rev Med Chem 2003;3:585–96.

85. Vicik R, Busemann M, Gelhaus C, Stief F, Scheiber J, Schmitz W, et al. Aziridine-based inhibitors of cathepsin L: synthesis, inhibition activity, and docking studies. ChemMedChem 2006;1:1126–41.

86. Nakao Y, Fujita M, Warabi K, Matsunaga S, Fusetani N, Mirazididine A a novel cysteine protease inhibitor from the marine sponge theonella aff. mirabilis. J Am Chem Soc 2000;122:10462–3.

87. Singh R, Zhou NE, Guo DQ, Kajita J, Cameron A, Parissima E, et al., inventors; Naja Pharmaceuticals Inc, assignee. 6-substituted amino-4-oxa-1-azabicyclo[3.2.0]heptan-7-one derivatives as cysteine protease inhibitors. European patent EP 0904284; 1997 Apr 9.

88. Ekiçi OD, Li ZZ, Campbell AJ, James KE, Asgian JL, Mikolajczyk J, et al. Design, synthesis, and evaluation of aza-peptide Michael acceptors as selective and potent inhibitors of caspases-2,-3, -6, -7, -8, -9, and -10. J Med Chem 2006;49:5728–49.

89. Barrett AJ, Kembhavi AA, Brown MA, Kirschke H, Knight CG, et al. Mutations in the proteolytic enzyme calpain 3 cause inclusion body myopathy, diabetes, and amyotrophic lateral sclerosis. J Med Chem 1996;39:5728–49.

90. Xu JG, Wang HD, Ding K, Lu YY, Li T, Wang JW, et al. Inhibition of cathepsin S produces neuroprotective effects after traumatic brain injury in mice. Mediat Inflamm 2013;2013:187873.

91. Ettari R, Nizi E, di Francesco ME, Dude M-A, Pradel G, Viñé R, et al. Development of peptidomimetics with a vinyl sulfone warhead as irreversibly faicalpin-2 inhibitors. J Med Chem 2008;51:988–96.

92. Sasaki T, Kikuchi T, Fukui I, Murachi T. Inactivation of calpain I and calpain II by specificity-oriented tripeptidyl chloromethyl ketones. J Biochem 1996;99:173–9.

93. Chatterjee S, Ator M, Foyezcko-Coyne D, Josef K, Wells G, Tripathy R, et al. Synthesis and biological activity of a series of potent fluoromethyl ketone inhibitors of recombinant human calpain 1. J Med Chem 1997;40:3820–8.

94. Angliker H, Wikstrom P, Rauber P, Shaw E. The synthesis of lysylfluoromethanes and their properties as inhibitors of trypsin, plasmin and cathepsin B. Biochem J 1987;241:871–5.

95. Eichhold TH, Hookfin EB, Taiwo YO, De B, Wehmeier KR. Isolation and quantification of fluorooacetate in rat tissues, following
Cysteine proteases as therapeutic targets

117. Shaw E. Cysteine proteinases and their selective inactivation. Adv Enzymol Relat Areas Mol Biol 1990;63:271–347.

118. Nakamura M, Yamaguchi M, Sakai O, Inoue J. Exploration of cornea permeable calpain inhibitors as anticaftaract agents. Bioorg Med Chem 2005;13:11371–9.

119. Ando R, Sakaki T, Morinaka Y, Takahashi C, Tamao Y, Yoshii N, et al. Cycloprenone-containing cysteine proteinase inhibitors. Synthesis and enzyme inhibitory activities. Bioorg Med Chem 1999;7:571–9.

120. Ando R, Morinaka Y, Tokuyama H, Isaka M, Nakamura E. A new class of proteinase-inhibitor. Cycloprenone-containing inhibitor of papain. J Am Chem Soc 1993;115:1174–5.

121. Battaglia F, Trinchese F, Liu SM, Walter S, Nixon RA, Arancio O, Calpain inhibitors, a treatment for alzheimer's disease. J Mol Neurosci 2003;20:357–62.

122. Cohen M, Brelter U, Albeck A. Peptidyl cycloprenonenes: reversible inhibitors, irreversible inhibitors, or substrates of cysteine proteases? Protein Sci 2013;22:788–99.

123. Li ZZ, Patil GS, Golubski ZE, Hori T, Tehrani K, Foreman JE, et al. Peptide alpha-keto ester, alpha-keto amide, and alpha-keto acid inhibitors of calpains and other cysteine proteases. J Med Chem 1993;36:3472–80.

124. Bartus RT, Hayward NJ, Elliott PJ, Sawyer SD, Baker KL, Dean RL, et al. Calpain inhibitor AK295 protects neurons from focal brain ischemia. Effects of postocclusion intra-arterial administration. Stroke 1994;25:2265–70.

125. Lee KS, Seo SH, Lee YH, Kim HD, Son MH, Chung BY, et al. Synthesis and biological evaluation of chromone carboxamides as calpain inhibitors. Bioorg Med Chem Lett 2005;15:2857–60.

126. Zhang Y, Jung SY, Jin C, Kim ND, Gong P, Lee YS. Design and synthesis of 4-aryl-4-oxobutanoic acid amides as calpain inhibitors. Bioorg Med Chem Lett 2009;19:502–7.

127. Kling A, Hornberger W, Mack H, Moeller A, Nimmrich V, Seemann D, et al. Cyclic ketone inhibitors of the cysteine protease cathepsin K. J Am Chem Soc 2008;130:6404–10.

128. Leung-Toung R, Wodzinska J, Li WR, Lowrie J, Kukreja R, Desilets D, et al. 1,2,4-thiadiazole: a novel cathepsin B inhibitor. Biochem Biophys Res Commun 1999;260:481–6.

129. Mack H, Kling A, Jantos K, Moeller A, Hornberger W, Hutchins CW, et al., inventors; AbbVie Inc., AbbVie Deutschl and GmbH & Co. KG, assignees. Carboxamide compounds and their use as calpain inhibitors. United States Patent 8283633; 2012 Oct 9.

130. Kling A, Jantos K, Mack H, Moeller A, Hornberger W, Lao YB, et al., inventors; AbbVie Inc., AbbVie Deutschland Co. KG, assignees. Carboxamide compounds and their use as calpain inhibitors IV. United States Patent 8598211; 2013 Dec 3.

131. Maryanoff BE, Costanzo MJ. Inhibitors of proteases and amide hydroxides that employ an a-ketoheterocycle as a key enabling functionality. Bioorg Med Chem 2008;16:1562–95.

132. Tao M, Bihovsky R, Kaiser JC. Inhibition of calpain by peptidyl heterocycles. Bioorg Med Chem Lett 1996;6:3009–12.

133. Palmer JT, Hirschtein BL, Chun H, McCarter J, Jone JW, Yu ZW, et al. Keto-1,3,4-oxidiazoles as cathepsin K inhibitors. Bioorg Med Chem Lett 2006;16:2909–14.

134. Liu H, Tully DC, Chatterjee A, Alper PB, Woodmansee DH, Mutnick D, inventors. IRM LLC, assignee. Compounds and compositions as cathepsin S inhibitors. United States Patent 6977256; 2005 Dec 20.

135. Marquis RW, Ru Y, Zeng J, Trout REL, LoCastro SM, Gribble AD, et al. Cyclic ketone inhibitors of the cysteine protease cathepsin K. J Med Chem 2001;44:725–36.
137. Stroup GB, Lark MW, Veber DF, Bhattacharyya A, Blake S, Dare LC, et al. Potent and selective inhibition of human cathepsin K leads to inhibition of bone resorption in vivo in a nonhuman primate. J Bone Miner Res 2001;16:1739–46.

138. Wijkmans J, Gossen J. Inhibitors of cathepsin K: a patent review (2004-2010). Expert Opin Ther Pat 2011;21:1611–29.

139. Asaad N, Bethel PA, Coulson MD, Dawson JE, Ford SJ, Gerhardt S, et al. Dipeptidyl nitrile inhibitors of cathepsin L. Bioorg Med Chem Lett 2009;19:4280–3.

140. Bethel PA, Gerhardt S, Jones EV, Kenny PW, Karoutchi GI, Morley AD, et al. Design of selective cathepsin inhibitors. Bioorg Med Chem Lett 2009;19:4622–5.

141. Falgueyret JP, Oballa RM, Okamoto O, Wesolowski G, Aubin Y, Rydzewski RM, et al. Novel, nonpeptidic cyanamides as potent and reversible inhibitors of human cathepsins K and L. J Med Chem 2001;44:94–104.

142. Yadav MR, Shinde AK, Chouhan BS, Giridhar R, Menard R. Biphenyl Derived Thiamides as Calpain Inhibitors. United States Patent 7476754; Jan 13 2009.

143. Herradon, B., Alonso, M., Benito, E., Chana, A., Montero, A. Solid-phase combinatorial synthesis of peptide-biphenyl hybrids as calpain inhibitors. Patent 2004-2010.

144. Cai JQ, Bennett DJ, Rankovic Z, Dempster M, Fradera X, Gillespie J, et al. 6-Phenyl-1H-imidazo[4,5-c]pyridine-4-carbonitrile as cathepsin S inhibitors. Bioorg Med Chem Lett 2010;20:4477–50.

145. Liu S, Yin F, Zhang JN, Qian YM. The role of calpains in traumatic brain injury. Brain Inj 2014;28:133–7.

146. Sribnick EA, Matzelle DD, Banik NL, Ray SK. Direct evidence for calpain involvement in apoptotic death of neurons in spinal cord injury in rats and neuroprotection with calpain inhibitor. Neurochem Res 2007;32:2210–6.

147. Adamiec E, Mohan P, Vonsattel JP, Nixon RA. Calpain activation in neurodegenerative diseases: confocal immunofluorescence study with antibodies specifically recognizing the active form of calpain 2. Acta Neuropathol 2002;104:92–104.

148. Nakashima H. Neuronal and microglial cathepsins in aging and age-related diseases. Ageing Res Rev 2003;2:367–81.

149. Markgraf CG, Velayo NL, Johnson MP, McCarty DR, Medhi S, Koehl JR, et al. Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. Stroke 1998;29:152–8.

150. Hook GR, Yu J, Sipes N, Pierschbacher MD, Hook V, Kingy MS. The cysteine protease cathepsin B is a key drug target and cysteine protease inhibitors are potential therapeutics for traumatic brain injury. J Neurotrauma 2014;31:515–29.

151. Hook VYH, Kingy M, Hook G. Inhibitors of cathepsin B improve memory and reduce β-amyloid in transgenic Alzheimer disease mice expressing the wild-type, but not the Swedish mutant, β-secretase site of the amyloid precursor protein. J Biol Chem 2008;283:7745–53.

152. Wam HI, Jacobsen JS, Rutkowski JL, Feuerstein GZ. Translational medicine lessons from flurizan's failure in Alzheimer's disease (AD) trial: implication for future drug discovery and development for AD. Clin Transl Sci 2009;2:242–7.

153. Marton Siklos et al.518

154. Cataldo AM, Hamilton DJ, Nixon RA. Lysosomal abnormalities in degenerating neurons link neuronal compromise to senile plaque development in Alzheimer disease. Brain 1994;260:68–80.

155. Grynszpan F, Griffin WR, Cataldo A, Katayama S, Nixon RA. Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease. Brain Res 1997;763:145–58.

156. Nixon RA. The calpains in aging and aging-related diseases. Ageing Res Rev 2003;2:467–81.

157. Hook V, Kingy M, Hook G. Cysteine protease inhibitors effectively reduce in vivo levels of brain β-amyloid related to Alzheimer's disease. Biol Chem 2007;388:247–52.

158. Yamashita T, Kohda Y, Tsuchiya K, Ueno T, Yamashita J, Yoshioka T, et al. Inhibition of ischaemic hippocampal neuronal...
death in primates with cathepsin B inhibitor CA-074; a novel strategy for neuroprotection based on ‘calpain-cathepsin hypothesis’. Eur J Neurosci 1998;10:1723–33.

180. Sahara S, Yamashima T. Calpain-mediated Hsp70.1 cleavage in hippocampal CA1 neuronal death. Biochem Biophys Res Commun 2010;393:806–11.

181. Sultana R, Perluigi M, Newman SF, Pierce WM, Cini C, Coccia R, et al. Redox proteomic analysis of carbonylated brain proteins in mild cognitive impairment and early Alzheimer’s disease. Antioxid Redox Signal 2010;12:327–36.

182. Yamashima T. Hsp70.1 and related lysosomal factors for necrotic neuronal death. J Neurochem 2012;120:477–94.

183. Zhu H, Yoshimoto T, Yamashima T. Heat shock protein 70.1 and related lysosomal factors for necrotic apoptosis by promoting mitochondrial release of cytochrome c. J Clin Invest 2008;116:500–63.

184. Luo CL, Chen XP, Yang R, Sun YX, Li QQ, Bao HJ, et al. Cathepsin B contributes to traumatic brain injury-induced cell death through a mitochondria-mediated apoptotic pathway. J Neurosci Res 2010;88:2847–58.

185. Guacciardi ME, Deussing J, Miyoshi H, Bronk SF, Svingen PA, Peters C, et al. Cathepsin B contributes to TNF-alpha-mediated hepatic cytochrome c and mitochondria-mediated caspase-9 activation. Cell Death Differ 2004;11:500–3.

186. Ishisaka R, Utsumi T, Yabuki M, Kanno T, Furuno T, Inoue M, et al. Activation of caspase-3-like protease by digitonin-treated lysosomes. FEBS Lett 1998;435:233–6.

187. Ishisaka R, Utsumi T, Kanno T, Arita K, Katunuma N, Akiyama J, et al. Participation of a cathepsin L-type protease in the activation of caspase-3. Cell Struct Funct 1999;24:465–70.

188. Yamada KH, Kozlowski DA, Seidl SE, Lance S, Wieschhaus AJ, Mercken M, et al. Inhibition of cathepsin B reduces beta-amyloid production in regulated secretory vesicles of neuronal chromaffin cells: evidence for a neuronal origin. Brain Res 1990;513:181–92.

189. Calpain-cathepsin hypothesis—a perspective review. Prog Neurobiol 2013;105:1–23.

190. Mercken M, Grenspan F, Nixon RA. Differential sensitivity to proteolysis by brain calpain of adult human tau, fetal human tau and PHF-tau. FEBS Lett 1995;368:10–4.

191. Yang LS, Kisezk-Reding H. Calpain-induced proteolysis of normal tau protein and tau associated with paired helical filaments. Eur J Biochem 1995;233:9–17.

192. Cataldo AM, Thayer CY, Bird ED, Wheelock TR, Nixon RA. Lysosomal protease antigens are prominently localized within senile plaques of Alzheimer’s disease: evidence for a neuronal origin. Brain Res 1990;513:181–92.

193. Haas U, Sparks DL. Cortical cathepsin D activity and immunolocalization in Alzheimer disease, critical coronary artery disease, and aging. Mol Chem Neuropharmacol 1996;29:1–14.

194. Gan L, Ye SM, Chu AL, Kii Y, Vilentic VA, et al. Cathepsin inhibition reduces beta-amyloid production in regulated secretory vesicles of neuronal chromaffin cells: evidence for cathepsin B as a candidate beta-secretase of Alzheimer’s disease. Brain Res 2005;1056:39–48.

195. Schilling S, Zuo YG, Ari A, Roberson ED, Sun BG, Chen J, et al. Antiamyloidogenic and neurprotective functions of cathepsin B: implications for Alzheimer’s disease. Neuron 2006;51:703–14.

196. Scheckter I, Ziv E. Kinetic properties of cathepsin D and BACE 1 indicate the need to search for additional beta-secretase candidate(s). Biol Chem 2008;389:313–20.

197. Rawlings ND, Barrett AJ. Chapter 404-introduction: the clans and families. In: Barrett AJ, editor. Handbook of proteolytic enzymes. 3rd ed. London: Academic Press; 2013, p. 1743–73.

198. Cynis H, Scheel E, Saito TC, Schilling S, Demuth H-U. Amyloidogenic processing of amyloid precursor protein: evidence of a pivotal role of glutaminyl cyclase in generation of pyroglutamat-modified amyloid-beta. Biochem Biophys Res Commun 2008;37:7405–13.

199. Schilling S, Lauber T, Schopp M, Manhart S, Scheel E, Bohm G, et al. On the seeding and oligomerization of pGlu-amyloid peptides (in vitro). Biochemistry 2006;45:12393–9.