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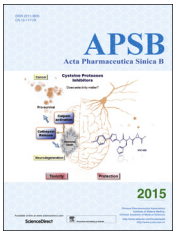

REVIEW

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 caspases, 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, Ca^{2+}/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, neurofibrilliary 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[1]. 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.[2] and earlier by Hernandez and Roush[3]. A review specific to cathepsin B inhibitors has also been published by Frlan and Gobec[4]. 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[2]. 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 pH_{max} 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[5,6].

The crystal structure of a number of cathepsins has been determined, among them cathepsin B[7]. 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[8]. The occluding loop has been targeted for the design of non-electrophilic cathepsin B inhibitors[9]. 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[10]. Table 1 shows residue preference of cathepsin B in peptide substrates in each position[11,12]. 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[13,14]. The first reports characterizing members of the enzyme family emerged in 1964, naming the enzyme calcium-activated neutral protease (CANP)[15–17]. .

Table 1 Cathepsin B: residue preference in peptide substrates in each position[11,12].

Unprimed[a]	Preference	Primed[a]	Preference
P1	Gly > Ala, Met, Gln	P1′	Phe > Gly
P2	Val > Phe, Tyr	P2′	Val, Ile > Gly, Thr
P3	Gly > Lys, Phe	P3′	Gly

[a]See Fig. 1 for depiction of primed and unprimed sites. Ala, alanine; Gln, glutamine; Gly, glutamine; Ile, isoleucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Tyr, tyrosine; Val, valine.

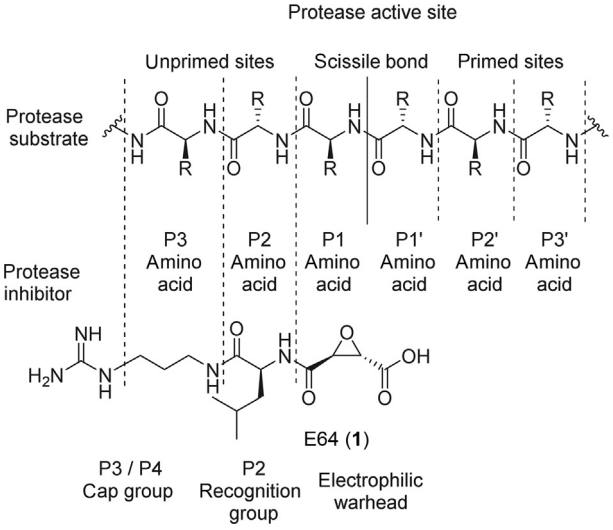

Figure 1 Nomenclature of primed and unprimed amino acid residues in protease substrates and inhibitors.

The enzymes consist of a catalytic subunit (82 or 80 kDa for calpains 1 and 2, respectively) and a Ca^{2+} binding subunit (28 kDa)[18]. The enzymes are unique among cysteine proteases in that the cytosolic proenzyme is activated by Ca^{2+} ions, inducing a conformational change. This change drives spatial proximity of the catalytic triad to the regulatory subunit, domain I, and subsequent autocatalytic cleavage[19]. 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 Ca^{2+} for activation, respectively[18]. The presence of phospholipids or phosphoinositides can decrease the Ca^{2+} concentration required for the activation of calpain 2[20,21]. 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

Table 2 Calpain: residue preference in peptide substrates at each position[41].

Unprimed	Preference	Primed	Preference
P1	Leu = Phe	P1′	Met > Ala > Arg
P2	Leu > Val	P2′	Glu
P3	Phe > Leu > Pro	P3′	Arg > Lys
P4	Phe	P4′	No specificity
P5	Pro	P5′	No specificity

Leu, leucine; Arg, arginine; Glu, glutamic acid; Leu, leucine; Pro, proline.

cellular machine under physiological conditions. Constant over-activation of calpain and other proteolytic enzymes, however, causes excessive protein degradation and neuronal death[22]. 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[23]. Transcription factors (c-Jun, c-Fos[24]) 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[25]. CREB is a key protein in synaptic plasticity, impaired activation of which is a key contributor to pathogenesis of Alzheimer's disease (AD)[26–29]. 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 familial AD[30]. 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[31,32].

The peptide sequence of the endogenous, specific inhibitor, calpastatin, is known and the inhibition mechanism has been elucidated[33,34]. 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[35]. Calpastatin is specific for the catalytically active form of calpain, bound to Ca^{2+}, 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[36]. 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[37,38]. Nevertheless, upregulation or decreased degradation of calpastatin is a therapeutic target in AD[39,40]. Table 2 shows residue preference of calpain in peptide substrates at each position[41].

Abnormal activation or dysregulation of calpains has been linked to a number of pathological conditions. The increased intracellular Ca^{2+} 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)[42,43], Huntington's disease[44,45], multiple sclerosis[46–48], and amyotrophic lateral sclerosis (ALS)[49,50]. Calpain 10 and the gene encoding it have been linked to type 2 diabetes mellitus[51,52]. A mutation causing loss of function of calpain 3 is believed to be responsible for limb-girdle muscular dystrophy[53].

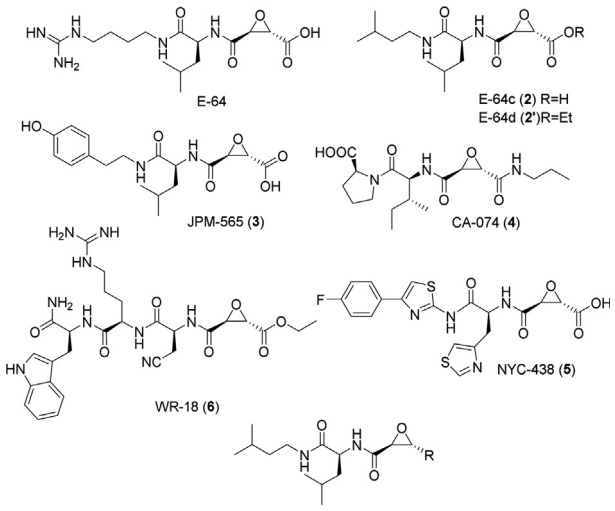

Figure 2 Structures of epoxysuccinate cysteine protease inhibitors.

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[41].

3. Electrophilic warheads for Cys protease inhibitors

3.1. *Irreversible covalent inhibitors*

Electrophiles that alkylate, acylate, phosphonylate or sulfonylate 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[54]. vinyl sulfonates, diazomethyl ketones and fluoro- or chloromethyl ketones[55–57]. 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[57]. Moderation of the reactivity of halomethyl ketones led to the development of acyloxymethyl ketones and other activated ketones such as aryloxymethyl, sulfonium methyl and ketoheterocycles. Other examples are vinyl ketones, vinyl esters and vinyl sulfones, which provide alternate Michael acceptor electrophiles[58,59]. Diazomethylketones have been explored and selectivity among different cathepsins attempted by the use of an appropriate recognition group[60–62].

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[63]. Total synthesis of E-64 soon followed[64]. 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[65,66]. The epoxide irreversibly modifies the active site Cys, forming a thioether bond[67,68]. 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.[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[70]. Calpain inhibitor reviews have appeared[41,71–73].

The chemical space around the P′ substituent of epoxide-containing peptidomimetics has been explored by Meara et al.[74,75]. 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: $CH_2OH < COCH_3 < COOR < CONH_2 < H < CONHOH < COOH$[74] (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[76]. E-64c itself had too poor cell permeability to inhibit calpain in intact platelets[76]. Other amide derivatives of E-64c that extended into the P′ site were weak calpain inhibitors compared to the free acid[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 dithioerythritol (DTT)[77,78]. The selectivity and bioavailability of epoxysuccinates was improved by substituting heterocyclic analogs for His at the P2 recognition group position by Schiefer et al.[79], 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[80]. Cyclic sulfates have been developed that show selectivity for cathepsin B over calpain, presumably due to the steric hindrance in the calpain active site[81,82]. Other Cys protease inhibitors containing oxiranes, thiiranes and aziridines were reviewed by Schirmeister et al.[83] Vicik et al.[84] 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 $K_i = 13$ nmol/L and cathepsin B $K_i = 9.4$ µmol/L (**13**, Fig. 3). This molecule conspicuously had an activity towards

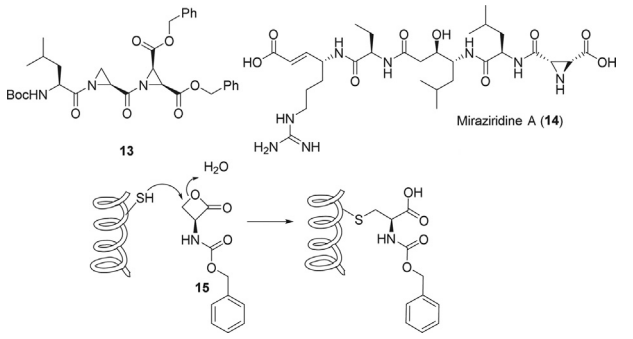

Figure 3 Structures of aziridine and *β*-lactone cysteine protease inhibitors.

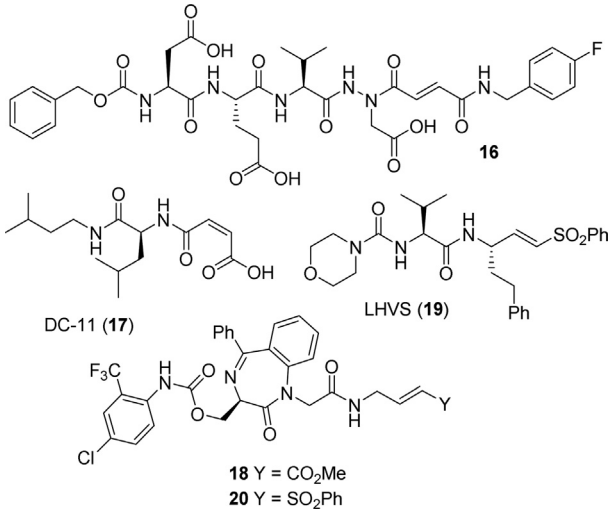

Figure 4 Structures of Michael acceptor warheads in cysteine protease inhibitors.

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 µmol/L[85], and both a reactive aziridine and less reactive Michael acceptor *α,β*-unsaturated carboxylate as terminal electrophiles.

β-Lactone and *β*-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[83]. A series of 6-substituted oxapenams was developed with the more potent inhibitor having 4 nmol/L potency against cathepsin L and good selectivity versus cathepsin B[86].

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[87]. Adducts of caspases with these Michael acceptors formed by 1,4-conjugate addition have been identified by X-ray

crystallography[87]. The fumaric acid derivative of E-64c (DC-11, **17**; Fig. 4) is 1000-fold less potent as an inhibitor of calpain 1 than E-64c[88,89], and is a weak irreversible inhibitor of cathepsins B and L[88]. 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)[58].

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 in the case of P2 = Leu[90]. LHVS (**19**, Fig. 4) has been used as a selective cathepsin S inhibitor *in vivo* and *in vitro*, to support a role for cathepsin S in TBI[91]; however, K_i = 0.40, 3.4 and 4.7 μmol/L for cathepsins S, B, and L, respectively[90]. The vinyl sulfone group was successfully used in several falcipain 2 inhibitors (**20**, Fig. 4)[92].

3.1.4. *Halomethyl ketones*

Developed as an His-selective alkylating agent, the chloromethyl ketone warhead is chemically reactive and alkylates reactive cysteine residues. The compound Z-Leu-Leu-Phe-CH_2Cl (**21,** Fig. 5) was reported as a calpain inhibitor with moderate potency[93]. Fluoromethyl ketone inhibitors were developed after the synthetic methodology became available[55]. The direct analog of the aforementioned chloromethyl ketone (**22**, Fig. 5) is a potent inhibitor of human recombinant calpain 1[94]. The chemical space around the "cap" group was explored, resulting in examples of inhibitors selective towards calpain 1 *vs.* cathepsin B. Fluoromethyl ketones are remarkably unreactive towards general thiol nucleophiles such as GSH[95]; however, the potential of fluoroacetate formation through metabolism has hindered clinical development[96]. Both fluoromethyl and chloromethyl ketones have been reported as potent inactivators of cathepsin B with the correct recognition group[55,97].

3.1.5. *Diazomethyl ketones*

Pioneering work on this warhead by Shaw and others[98–100] showed that diazomethyl ketones are irreversible inhibitors of papain. The inhibition of calpain and the cathepsins depends on the recognition peptide sequence used. Diazomethyl ketones are cell permeable and sufficiently stable in the presence of thiol-containing reducing agents such as DTT and mercaptoethanol[101]. Selectivity was targeted for cathepsin isozymes[60–62]. A diazomethyl ketone analog (Z-Leu-Leu-Tyr-CH_2N_2, **23**; Fig. 5) of the aforementioned halomethyl ketones was reported with potency for calpain 1 inhibition intermediate between cathepsin L and cathepsin B.[61]

3.1.6. *Acyloxymethyl and other activated ketones*

Acyloxymethyl ketones are reported to have low reactivity towards GSH[102]. The cathepsin B inhibition of a series of acyloxymethyl ketones (Z-Phe-Ala-CH_2OCO-R) was inversely correlated with the pK_a of the carboxylate leaving group, with the 2,6-bis-trifluoromethylbenzoate (**24**, Fig. 5) having the highest potency[103]. The compounds also show potent inhibition of cathepsins L and S but not calpain 1. An alternative approach reported a sulfanylmethyl ketone (**25**, Fig. 5) that was a potent inhibitor of cruzain with good selectivity (cruzain K_i ~ 0.9 nmol/L, cathepsin B ~ 700 nmol/L, cathepsin L ~ 28.8 nmol/L)[104,105]. Unlike acyloxymethyl ketones, sulfonium methyl ketone peptidomimetics, *e.g.*, Z-Leu-Leu-Phe-$CH_2S^+(Me)_2$ (**26**, Fig. 5), inhibited calpain 1 with high potency.

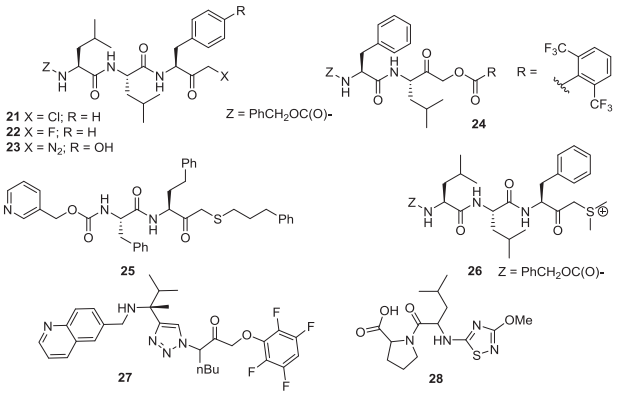

Figure 5 Structures of diazomethyl, acyloxy and other ketone cysteine protease inhibitors.

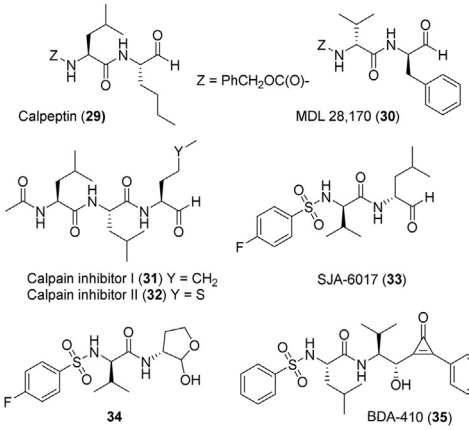

Figure 6 Structures of aldehyde and cyclopropenone inhibitors.

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[106]. Tetrafluoro-phenoxymethyl ketones (*e.g.* **27**) have been developed as potent cruzain inhibitors[107].

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[108].

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*

Since by definition, reversible covalent inhibitors do not yield a stable adducted enzyme, evidence for covalent mechanisms must

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[109–111]. Peptide aldehydes were isolated from *Streptomyces* strains and were found to inhibit calpain and other proteases[112]. The physicochemical characteristics of the compounds were improved by substituting the terminal amino acid for a hydrophobic cap group such as benzyloxycarbonyl, 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**)[115]. 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[116]. 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 prodrug 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 nucleophilic 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

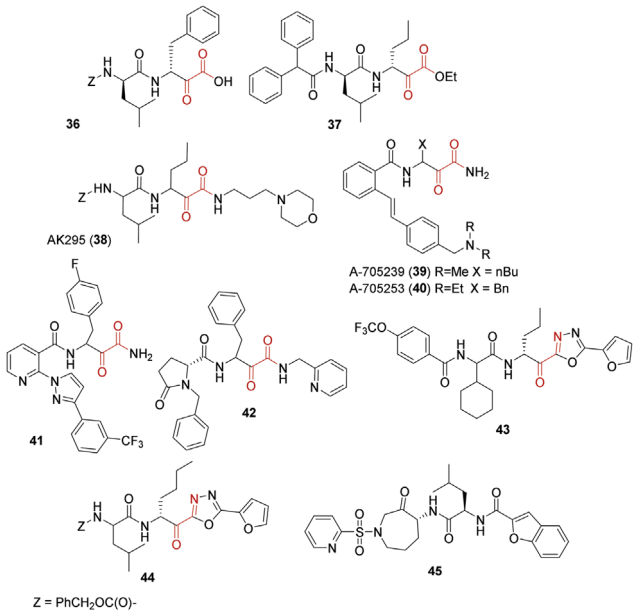

Figure 7 Structures of ketoamide and ketoheterocycle cysteine protease inhibitors.

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: $20<K_i<200$ 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[124].

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 chromenone derivatives were described, along with ring-opened 4-aryl-4-oxobutanoic 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 explored included bioisosteric 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[132]. Early examples, were developed by optimization of P′, P2, 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_i.[136] SB-357114 (**45**, Fig. 7) was reported to inhibit human cathepsin K with a K_i of 0.16 nmol/L, 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.

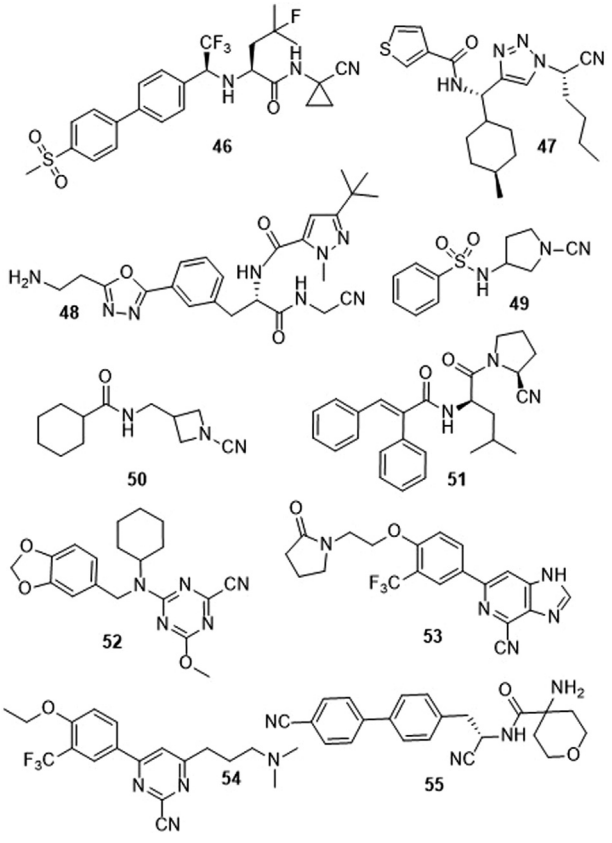

Figure 8 Structures of nitrile and carbodiimide inhibitors.

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, rhodesain; 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-1*H*-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 aminoacetonitrile 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-151746, **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

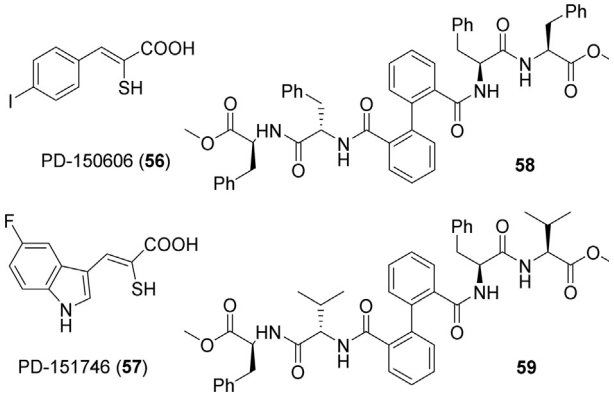

Figure 9 Allosteric inhibitors of calpain.

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 *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]. CA-074 and a cathepsin B antibody reduced lung metastases in mice after the injection of human melanoma cells[160]. In a further mouse breast cancer model, CA-074 but not JPM-OEt, was found to decrease tumor invasion, neovascularization, and bone metastases[161]. The bioavailability of cathepsin inhibitor drug generated by the prodrug, JPM-OEt, is problematic[162].

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[163]. 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[164,165]. Calpain 1 is highly expressed in neurons[166] and calpain 2 in glial cells[167]. 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[168]. 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[165]. 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[91]. 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]. Neurofibrillary 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[171,177,178].

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.[187] Interestingly, pro-apoptotic activation was profoundly suppressed by cysteine protease inhibitors leupeptin and E64.[188] However, CA-074, an epoxysuccinate cathepsin B inhibitor, did not inhibit digitonin-mediated caspase activation[188], indicating that not only lysosomal cathepsin B but also other lysosomal cysteine protease are involved in the cascade leading to neuronal death.[189] 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[184,185].

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[186]. 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[170]. 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[170], indicating a neuroprotective role for "off-target" inhibition of calpains, which are also validated drug targets in TBI[164,190]. Indeed, brain calpain activity spikes within 24 h of trauma[191,192], and E-64d administration has been shown to reduce calpain activity and provide neuroprotection after trauma[193,194]. 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 metallopeptidase-9 (MMP-9)[195], a known contributor to TBI[196,197], 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[198,199]. It is therefore logical to propose that the calpain-cathepsin cascade, associated with ischemic neuronal death, contributes to AD pathogenesis[200]. In AD brains, calpain 1 activity is increased 7-fold compared to age-matched brains[201]. Amyloid precursor protein (APP) and amyloid β (Aβ) were also reported to induce calpain activation[202,203], and evidence exists for reciprocal processing by calpain1 of APP[204] and tau proteins[205,206]. Activated calpain was observed to occur in neurofibrillary tangles, senile plaques (SP), and dystrophic neuritis.[23] Similarly, cathepsin D was observed to be localized extracellularly within senile plaques by immunoassay.[207,208] 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[208].

In activated microglia, cathepsin B was claimed to be a key player in A$\beta_{1\text{-}42}$ induced neuronal death[209]. 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[210]. E-64d treatment rescued memory function, and decreased brain A$\beta_{1\text{-}40}$/A$\beta_{1\text{-}42}$ and amyloid plaque neuropathology in AD animal models expressing human APP containing the wild-type β- and London mutant γ-secretase site (APPLon) sequences[171,177]. Cathepsin B inhibition had no effect on Aβ pathology in mice expressing APP containing the Swedish mutant β-secretase site sequence (APPSwe)[171,211]. Nevertheless, BDA-410 and E-64 improved memory deficits in APPSwe mice[30] 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[212]; importantly, relevant cathepsin D levels are about 280-fold greater than BACE-1[212]. In the APP_{Swe} 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[213]. 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[214]. In turn, cathepsin B may be involved in the production of pGlu forms of Aβ[214] that aggregate at accelerated rates[215].

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.

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