Calpain research for drug discovery: challenges and potential

Yasuko Ono[1], Takaomi C. Saido[2] and Hiroyuki Sorimachi[1]

Abstract | Calpains are a family of proteases that were scientifically recognized earlier than proteasomes and caspases, but remain enigmatic. However, they are known to participate in a multitude of physiological and pathological processes, performing 'limited proteolysis' whereby they do not destroy but rather modulate the functions of their substrates. Calpains are therefore referred to as 'modulator proteases'. Multidisciplinary research on calpains has begun to elucidate their involvement in pathophysiological mechanisms. Therapeutic strategies targeting malfunctions of calpains have been developed, driven primarily by improvements in the specificity and bioavailability of calpain inhibitors. Here, we review the calpain superfamily and calpain-related disorders, and discuss emerging calpain-targeted therapeutic strategies.

We dedicate this Review to our late mentor Koichi Suzuki, who made tremendous scientific contributions as the world leader in calpain research for four decades, led us into the world of calpains and died too soon on 20 April 2010 aged 71 years.

Calpains, discovered in 1964 (REF. 1), are clan CA family C02 cysteine proteases (EC 3.4.22.17) defined by a well-conserved cysteine protease domain called the CysPc motif[2–4] (FIG. 1). The word calpain combines 'cal' and 'ain', which are respectively derived from calcium and cysteine proteases such as papain and legmain[5]. Calpains were previously called Ca^{2+}-activated neutral proteases[6,7].

Although there are vast numbers of proteases and proteolytic complexes in biological systems, the calpains are some of the very few that are directly activated by Ca^{2+}, a primary second messenger in signal transduction. In addition, calpains are modulator proteases that perform limited proteolysis to modulate rather than abolish the function of their substrate. Two key biological elements, Ca^{2+} and proteolysis, are embodied in calpains, and basic and clinical researchers often find themselves trying to fit calpains into their subject of interest. The identification of calpains as aggravating factors in human diseases can be attributed to such scientific curiosity. However, although the vital roles of calpains in various biological systems are clear, their mechanistic features remain poorly understood. Owing to their modulatory nature and lack of clear cleavage sequence specificity, calpains appear to have less precise activity and to be less amenable to systematic examination than proteases such as caspases and those involved in the ubiquitin–proteasome system. Consequently, the biological functions and therapeutic potential of calpains remain relatively unexplored.

Calpain-related disorders have a substantial impact on society. Although some diseases are caused by genetic defects, many are lifestyle-related[8] or caused by infection with a pathogenic microorganism[9–13]. These diseases are of growing concern worldwide from both human health and economic perspectives. Disorders for which calpains may serve as therapeutic targets include neurodegenerative disorders[14–22], cardiovascular diseases[23], ischaemic disorders[24], arterial sclerosis[25,26] and cancers[27–30]. Additional disorders include cataracts[31], muscular dystrophies[32], gastric ulcer[33], vitreoretinopathy[34], oesophagitis[35,36], pulmonary fibrosis[37], diabetes[38,39], malaria[40,41], trypanosomiasis[42], schistosomiasis[10], candidiasis[43] and periodontitis[12]. In most cases of calpain-related disease, calpain activity is elevated and aggravates symptoms. For example, transient cerebral ischaemia caused by various insults results in calpain activation that leads to neuronal cell death[44].

Some calpain inhibitors have already been developed for therapeutic applications. For example, the *Aspergillus*-derived calpain inhibitor E-64d (also called loxistatin, aloxistatin, rexostatine, EST and Estate) was used in clinical trials for Duchenne muscular dystrophy (DMD)[45], and the α-ketoamide inhibitor A-705253 (also known as BSF 419961 and CAL-9961) has been tested as a treatment for Alzheimer disease (AD)[46]. More recently, it was recognized that restoration or compensation of calpain activity could ameliorate diseases such as limb-girdle muscular dystrophy type 2A (LGMD2A)[47] and spastic

Clan
Peptidases and their proteinaceous inhibitors are systematically classified by the online MEROPS database. A clan is composed of multiple families, each of which corresponds to a group of orthologous peptidases. Members of a clan share similar primary and tertiary structures. This classification system complements and extends the previously established enzyme classification system, which includes all of the enzymes.

[1]Calpain Project, Department of Advanced Science for Biomolecules, Tokyo Metropolitan Institute of Medical Science (IGAKUKEN), 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156–8506, Japan.
[2]Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, 2–1 Hirosawa, Wako, Saitama 351–0198, Japan.
Correspondence to H.S. and T.S. sorimachi-hr@igakuken.or.jp; saido@brain.riken.jp

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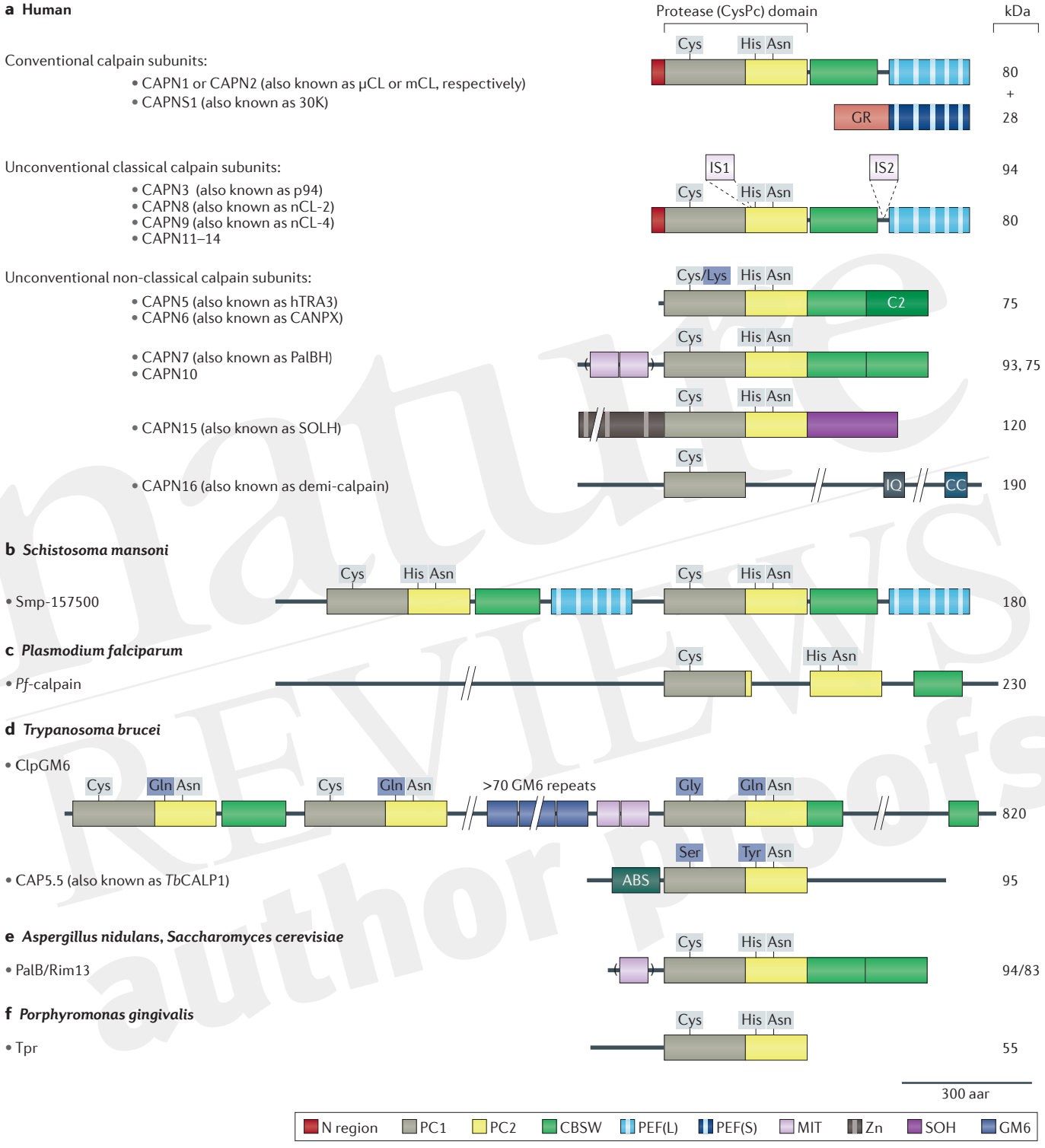

Figure 1 | **Schematic structure of calpains. a** | In vertebrates, calpain-1 and calpain-2 are heterodimers of a large catalytic subunit (CAPN1 and CAPN2, respectively) and a small regulatory subunit (CAPNS1). Calpain-1 and calpain-2 are called conventional calpains, with the remaining calpains known as unconventional calpains (avian calpain-11 (CAPN11–CAPNS1) may also be called a conventional calpain). Calpains are classified into two types on the basis of their structure: classical, with domain structures that are identical to those of CAPN1 and CAPN2, and non-classical. **b**–**f** | Among the non-human calpains shown, only Smp-157500 (the carboxyl-terminal half is known as Smp-80) of *Schistosoma mansoni* is a classical calpain. *Plasmodium falciparum* and *Porphyromonas gingivalis* each have only one calpain, whereas *S. mansoni*, *Trypanosoma brucei* and *Aspergillus nidulans* have 7, 18 and 2 calpains in total, respectively. The number of repetitions of GM6 antigen repeat unit could not be determined owing to an incomplete genomic sequence. ABS, all β-strand structure (found among several glycosyl hydrolases); CBSW, calpain-type β-sandwich domain; CysPc, cysteine protease domain, calpain-type; GR, glycine-rich domain; IQ, calmodulin-interacting domain; IS, insertion sequence; MIT, microtubule-interacting and transport domain; PC, protease core; PEF, penta-EF-hand domain; SOH, SOL-homology domain; Zn, zinc-finger motif.

paraplegia[48]. Therefore, calpain-targeting strategies now encompass two key approaches: calpain inhibition and calpain activation (either directly or by replacement).

This Review seeks to address how the wealth of discoveries gained from calpain research can be translated into strategies for combating various disorders. In this regard, we first describe the current state of calpain research and discuss the pathological roles of calpain activity. We then assess various therapeutic strategies that target calpains as defined by three major approaches: first, the inhibition of human calpains that aggravate symptoms; second, inhibition of the calpains of parasites or pathogenic microorganisms to disrupt the invasion, growth and/or egress of the organism; and, last, complementation of calpain functions by gene therapy or gene editing to correct or compensate for a defective calpain. Importantly, the challenges faced in therapeutic modulation of calpain activity are discussed.

The calpain superfamily

Human calpains. Calpains exist in almost all eukaryotes and some bacteria, whereas calpains are not present in very few fungi and no calpain gene has been found so far in the genome of any archaea[49–52]. Various motifs or domains (>40) occur together with the CysPc motif[51,52]. Humans express 15 calpain genes, *CAPN1* to *CAPN16* except for *CAPN4*, each of which generates one or more transcripts and constitute a superfamily of more than 50 molecular species (for example, *CAPN3* has more than 10 variants)[4,53]. Mutations in individual calpain genes are associated with lethality or disorders known as calpainopathies, indicating the importance of calpains in mammalian life and health[4,53] (see below and TABLE 1). In non-mammals, calpains have also been genetically shown to be involved in various biological phenomena, such as optic neuron development in fruit flies, sex determination in nematodes, alkaline environment adaptation in fungi and yeasts, and germ cell generation in plants[2,4,49,54,55].

Calpain-1 and calpain-2, known as the conventional calpains, are the most ubiquitous and well-studied calpain family members, and are activated *in vitro* by micromolar and millimolar concentrations of Ca^{2+}, respectively[2]. They modulate the structures and functions of their substrates by limited proteolysis[2,50]. There is a long-standing paradox regarding the mechanisms of calpain-1 (also known as μ-calpain) and calpain-2 (also known as m-calpain)activation: given that physiological intracellular Ca^{2+} concentrations reach micromolar levels at most, calpain-1 was thought to be used most widely *in vivo*. However, reverse genetics has shown that CAPN1-deficient mice are apparently normal[56], whereas CAPN2 deficiency induces embryonic lethality[57,58], suggesting that calpain-2 has more important functions than calpain-1 *in vivo*. The reason for the discrepancy between the biochemistry and the biology of calpain-1 and calpain-2 remains largely elusive.

Among the unconventional calpain subunits, CAPN5, 7, 10, 13 and 15 also exist in most cells (called ubiquitous calpains for descriptive purposes), whereas CAPN3, 6, 8, 9, 11, 12, 14 and 16 are expressed predominantly in particular tissues and organs (tissue-specific calpains)[4,50]. The tissue-specific calpains, when defective, cause disorders specific to the tissues in which they are expressed. For example, pathogenic mutations in *CAPN3*, which is predominately expressed in skeletal muscle, result in LGMD2A[32] (TABLE 1). By contrast, the ectopic expression of CAPN3 in the heart, another tissue composed of striated muscle, is lethal, highlighting the need for tightly regulating calpain expression in specific tissue and cellular environments[47,59]. Intriguingly, despite their presence in most tissues, pathogenic alterations in *CAPN1* and *CAPN5* lead to deficiencies in specific tissues (neuronal cells and eyes, respectively)[34,48].

Between 1964 and 1988, calpain research focused solely on conventional calpains. The first unconventional calpain was identified in 1989 (REF. 60), followed by the discoveries of others. Since the twenty-first century, unconventional calpains have become a critical topic in the calpain field[4,50]. An endogenous proteinaceous calpain inhibitor, calpastatin (CAST), has been found in mammals and birds, and adds another layer of complexity to the calpain system[61]. The inhibitory activity of CAST is specific for calpains, and it does not inhibit any other proteases[61] (see below).

Structures of calpains. The conventional calpains consist of a large catalytic subunit and a small regulatory subunit (FIG. 1). The large subunit (CAPN1 and CAPN2 for calpain-1 and calpain-2, respectively) has an amino-terminal anchor helix (N region), a CysPc domain composed of protease core 1 (PC1) and PC2 domains, a calpain-type β-sandwich (CBSW; previously known as C2-domain-like (C2L)) domain, and a penta-EF-hand (PEF)[62] domain (PEF(L)). The common small subunit CAPNS1 is composed of glycine-rich (GR) and PEF(S) domains (FIG. 1) and is essential for the stability of CAPN1 and CAPN2. Genetic disruption of *Capns1* in mice inactivates both calpain-1 and calpain-2 (REFS 58,63). CAPN3, 8, 9 and 11–14 have domain structures identical to those of CAPN1 and 2, and are called classical calpain subunits, whereas the remaining non-classical calpain subunits have another motif (or motifs) in place of the PEF domain. CAST, the calpain-specific inhibitor protein, has several splicing variants, the longest of which consists of N-terminal non-inhibitory regions and four repeated calpain-inhibitory domains, which are each composed of three subdomains: A, B and C[61]. Subdomain B interacts with the CysPc domain, whereas subdomains A and C, which form α-helices, bind to PEF(L) and PEF(S) of the conventional calpains, respectively, and stabilize the CAST–calpain complex[64,65].

The 3D structure of calpain-2 was the first to be reported[64–67]. So far, the only calpain for which the complete 3D structure has been solved is calpain-2; however, 3D modelling of other calpains has been accepted as a rational approach[68–71]. Four domains of CAPN2 align on the same surface, which spreads over an imaginary oval plane. In the absence of Ca^{2+}, PC1 and PC2 have open structures that do not form a catalytic triad[66,67]. Activation by Ca^{2+}, surprisingly, does not dramatically change the overall calpain structure; instead, one Ca^{2+}

CysPc motif
A protease catalytic domain of calpains. CysPc-containing proteases belong to the same clan as papain, but unlike the catalytic domain of papain the CysPc divides into two separate structures, protease core 1 (PC1) and PC2, in the absence of Ca^{2+}. Amino acid sequence comparisons suggest that all of the calpain species for which 3D structures are not yet solved share similarity in their CysPc domain.

α-Ketoamide inhibitor
A class of reversible inhibitors for cysteine, serine or threonine proteases that add an electrophile to these active site amino acid residues. Many inhibitors of this class have been developed by systematically replacing aldehyde moieties of known calpain inhibitors with α-ketoamide, and subsequently modifying other positions to improve effectiveness.

Calpainopathies
Diseases caused by a genetic defect in a calpain gene. The pathogenic mechanism can be either loss of function (for example, limb-girdle muscular dystrophy type 2A is caused by inactivating mutations in the *CAPN3* gene) or gain of function (for example, autosomal dominant neovascular inflammatory vitreoretinopathy is caused by an excessive activation of CAPN5 due to mutations).

Calpain-type β-sandwich
(CBSW). A domain whose 3D structure, but not primary sequence, shows overall similarity to the C2 domain, a calcium-binding motif found in protein kinase C, synaptotagmins and other calcium-related proteins. The CBSW domain has a role in substrate recognition.

Penta-EF-hand
(PEF). Among the proteins with Ca^{2+}-binding EF-hand motifs, those with five EF-hand motifs in tandem comprise the PEF family. The fifth EF region is often involved in homo- or heterodimerization. Classical calpains have a PEF domain, and hence also belong to the PEF family.

Table 1 | **Calpainopathies**

Gene (alternative names)*	Expression preference	Phenotype related to gene mutation
CAPN1, *Capn1*	All cells	Spastic paraplegia[48], platelet dysfunction[56] and spinocerebellar ataxia (hereditary also in dogs)[170,171]
Capn2	Most cells	Embryonic lethal[57,58]
Capns1	All cells	Embryonic lethal[63,263]
CAPN3, *Capn3*	Skeletal muscle	Various muscular dystrophies[32,161,264,265]
CAPN5, *Capn5*	Most cells	Vitreoretinopathy[34,165]
Capn6	Embryonic muscle	Hypergenesis[240]
CAPN7	Most cells	NP
Capn8	Gastrointestinal tract	Gastric ulcer[33]
Capn9	Gastrointestinal tract	Gastric ulcer[33]
CAPN10	Most cells	Type 2 diabetes[251]
CAPN11	Testes	NP
CAPN12	Hair follicles, skin	Congenital ichthyosis[266]
CAPN13	Most cells	NP
CAPN14	Oesophagus	Eosinophilic oesophagitis[35]
CAPN15 (*SOLH*)	Most cells	NP
CAPN16 (*ADGB*, *C6ORF103*)	Testes	NP

NP, not yet published for human or mouse gene. **CAPN* and *Capn* indicate human and mouse genes, respectively.

binds to each of the PC domains, which causes PC1 and PC2 to connect, forming the 'closed' active structure[64,65] (FIG. 2). Crystal structures of the active calpain-2–CAST complex show that the CAST amino acid residues that interact near the active site of calpain do so mostly through their peptide bond backbone atoms, whereas the side chains of CAST are mostly oriented outwards from the structure and do not engage in atomic interactions with calpain[64,65]. This preference for binding to the peptide bond backbone atoms may explain the apparently low amino acid sequence selectivity for calpain substrates.

The cleavage site of a protease substrate can be described as the bond between amino acid site P1 on the N-terminal side and P1′ on the carboxy-terminal side. The residues oriented away from the cleavage site are labelled P2, P3, and so on, and P2′, P3′ and so on. The site in the binding pocket of the protease that binds with P1 is called S1, and so on. In calpain substrates, P2, P1 and the primed sites interact with CysPc, whereas most of the unprimed sites in the N-terminal to P2 are recognized by CBSW[64,65] (FIG. 2). Together with the 3D structures of CysPc–inhibitor complexes[72–75], these findings indicate that the P2–S2 interaction between a conventional calpain and its substrate is prominent, similar to other clan CA proteases such as cysteine cathepsins, and that the primed sites are also important for the efficient and specific blockade of calpains by inhibitors.

A recent structural insight worth noting is that of the complex of calpain with its allosteric inhibitors. To date, an α-mercaptoacrylic acid derivative (PD150606) and an oligopeptide (LSEAL) have been proposed to exert an allosteric inhibitory effect on calpains[76–78]. Both molecules bind to hydrophobic clefts in the calpain PEF domains in a manner similar to the CAST subdomains A and C. However, a recent study showed that PD150606 inhibits calpains by directly interacting with the CysPc domain, and that LSEAL does not inhibit calpains *in vitro*[79]. As discussed below, the mode of inhibition by PD150606 remains elusive.

The 3D structure of calpain has revealed new strategies for inhibitor design. For example, the specific inhibition of calpain by CAST is enabled by the core inhibitory sequence within subdomain B of CAST, which is positioned beyond the active site[64,65]. This finding has led to a new concept for inhibitor design, in which molecules such as macrocyclic peptides are of interest. In addition, although the available structural data are currently limited, examining differences among the CysPc structures from different calpains is possible: for example, the domain rotations of PC1 and PC2 relative to each other[72,80,81] (FIG. 2). Such information can direct the design of specific inhibitors and/or substrates for each calpain species (BOX 1).

Substrate specificities of calpains. The identification of calpain substrates has revealed several signal transduction-related molecules for which proteolysis by calpain results in functional alterations. One interesting substrate is p35, which can be converted by calpain to p25, an activator of cyclin-dependent kinase 5 (CDK5) that may be involved in memory formation[82]. Another interesting substrate is interleukin-1α (IL-1α)[83], an inflammation-related cytokine in humans. IL-1β is secreted after its cleavage by caspase 1, whereas IL-1α is cleaved by calpain[84] and then transported out of the cytoplasm by an as yet unknown mechanism. In addition, cleavage of the MYC oncoprotein by calpain is a critical step in transforming the functions and localization of this key molecule in cancer cell survival[85–87]. The

CAPNS1
(Calpain small subunit 1). A PEF family member. A paralogue called CAPNS2 has an unknown function. *In vivo*, CAPNS1 is an essential component of the two conventional calpains, calpain-1 and calpain-2. However, *in vitro*, the cysteine protease domains isolated from CAPN1 and CAPN2 (mini-calpains) are functional as active proteases.

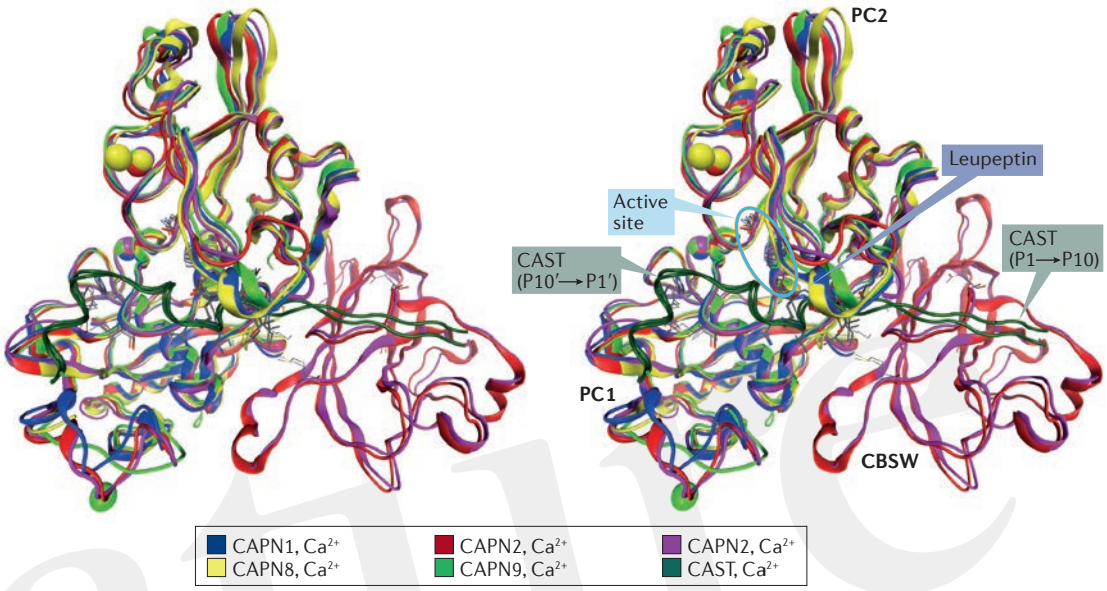

Figure 2 | **Cross-eye stereo view of CAPN1, 2, 8 and 9.** The cysteine protease (CysPc) domain of CAPN1, the large catalytic subunit of calpain-1 (RCSB Protein Data Bank (PDB) ID: 1TL9), CAPN8 (PDB ID: 2NQA) and CAPN9 (PDB ID: 2P0R) are shown in complex with leupeptin (shown by stick models) and Ca^{2+} (shown by balls). The CycPc and calpain-type β-sandwich (CBSW) domains of CAPN2 (PDB ID: 3BOW (red) and 3DF0 (pink)) in a complex with calpastatin (CAST; indicated by dark green tubes; right to left corresponds to amino terminus to carboxyl terminus) and Ca^{2+} were superimposed at the protease core 1 (PC1) and PC2 regions. The amino acid alignment (data not shown) according to these superimpositions indicates that the amino acid residues proximal to CAST are well conserved among CAPN1, 2, 3, 8 and 9, except that several amino acid residues in CBSW (corresponding to S3–S10) are divergent in CAPN3. Structures generated by MOE Ver.2015.10 (Chemical Computing Group Inc., Montreal, Quebec, Canada).

calpain-mediated cleavage of dysferlin, a gene product responsible for LGMD2B, also exemplifies the transformative nature of calpain. The C-terminal C2 domains of dysferlin are released by calpain in response to membrane injury, and these domains function as effectors in a membrane repair cascade[88,89]. Finally, one of the best-known calpain substrates is spectrin, an important membrane cytoskeletal maintenance protein (spectrin-α chain non-erythrocytic 1 is also known as α-fodrin). The calpain-dependent proteolysis of spectrin is closely associated with neuronal cell death[90]. Spectrin is proteolysed at a specific site by calpain-1 and calpain-2 (REFS 91,92), as well as by other calpains[93].

Elucidating substrate specificity at the molecular level has been a challenge in calpain research, although it is emerging as an attractive subject for bioinformatics studies. Initial studies at the protein and amino acid sequence levels revealed the limited nature of the proteolytic activity of conventional calpains[94]. A few years later, a position-specific scoring matrix analysis revealed the amino acid residue preference at each position around the cleavage site, which provided the first theoretical information regarding the substrate specificities of calpains[95]. Further studies were carried out using peptide libraries[74,96–98] and/or machine learning[98–102], which yielded calpain cleavage site predictors that were sufficiently accurate for practical use[98–102]. A comprehensive kinetics analysis of peptide-array substrates showed that in addition to P2, the P3′ and P4′ sites are important for the cleavage efficiency of calpains[98]. These features may provide directions for peptide-based inhibitor design.

Human disorders involving calpains

Calpain-associated diseases can be categorized into three types according to the way calpain is involved in their pathogenesis: those caused or exacerbated by human calpain activities (type 1; for example, neurodegenerative and cardiovascular disorders, ischaemia, cancers and cataracts); those caused by parasites or pathogenic microorganisms that use the host's and/or their own calpains for infection and survival (type 2; for example, malaria, trypanosomiasis, schistosomiasis, candidiasis and periodontitis); and calpainopathies caused by deficiencies in calpain genes (type 3; for example, muscular dystrophies, vitreoretinopathy, gastric ulcer and oesophagitis) (TABLES 1,2). Representative examples are described below.

Many studies of calpain-related disorders have used transgenic mice. For example, mice deficient in calpain-1 and/or calpain-2 and mutant mice that lack or overexpress CAST (*Cast*$^{-/-}$ and transgenic CAST mice, respectively)[23,59,103–105]. Together with antibodies that recognize activated CAPN1 (REF. 106) or calpain-cleaved spectrin[107], these mouse models have been valuable tools for revealing clear cause-and-effect relationships in neurodegenerative and other disorders. However, there is growing awareness that transgenic mice are an idealized and simplified experimental system and that the results obtained using these systems must be carefully interpreted.

Neurodegenerative disorders. Several reports have indicated that calpain may have a major aggravating role in the pathogenesis of AD[20,108–110]. For example, calpain-1 is hyperactivated in the AD brain[108], and calpain inhibitors

Box 1 | **Improving the specificity of human calpain inhibitors**

Most calpain inhibitors (Supplementary information S1,S2 (figure, table)) are not specific and block other proteases such as cysteine cathepsins. Calpastatin (CAST) is the only absolutely specific inhibitor for classical calpains: calpain-1 (CAPN1–CAPNS1) and calpain-2 (CAPN2–CAPNS1), calpain-9 (CAPN9–CAPNS1), calpain-8 (CAPN8)$_2$) and calpain-8/9 (CAPN8–CAPN9; also known as G-calpain)[2,33,61,253]; however, it does not inhibit calpain-3 (CAPN3)[68,254]. The mini-calpain mutants cysteine protease (CysPc)-only CAPN1 and CAPN2 are also not effectively inhibited by CAST[255,256]. The calpain amino acid residues proximal to the bound CAST are well conserved among CAPN1, 2, 3, 8 and 9, except that several amino acid residues of the calpain-type β-sandwich (CBSW) domain that contact CAST at the P3–P10 sites are divergent in CAPN3. The alignment of the CBSW domain relative to the CysPc domain may also be different between CAPN3 and other calpain subunits. Thus, the CAST–CBSW interaction appears to be key for CAST specificity.

The existence of CAST indicates that there is a solution to developing absolutely specific calpain inhibitors. A relatively long peptide bridging CysPc and CBSW may be required. Once an inhibitor is discovered, issues such as its stability and bioavailability *in vivo*, membrane penetrability and production cost need to be examined. With regard to these issues, secondary-structure-fixed inhibitors (CAST peptidomimetics and macrocyclic peptides) discussed in the main text[188,190,192] and calpain-derived inhibitory peptide[193] appear to be promising. Currently, although the specificity of these peptide inhibitors is satisfactory, their stability *in vivo*, bioavailability, cost to synthesize and/or cell penetrability need to be improved for their application as therapeutic agents. Among these inhibitors, β-strand-fixed macrocyclic inhibitors can be relatively easily applied for proteases other than calpains (such as cathepsins L and S, and proteasomes) by substituting amino acid residues[191]. This approach may be applied for isoform-specific calpain inhibitors (see below).

Almost all calpain inhibitors are active site-directed, which is one of the reasons for their poor specificity for calpains. Because protease core 1 (PC1) and PC2 need to connect to be active, inhibiting this connection, for example, by an intercalation between PC1 and PC2, may result in supremely specific inhibitors for calpains. Similarly, other allosteric inhibitors discussed in the main text are also worth pursuing[79,195,196]. Alternatively, when the involvement of cysteine proteases other than calpains cannot be ruled out owing to a lack of inhibitor specificity, inhibitors specific for cysteine cathepsins can be tested for their ability to suppress disorders that are ameliorated by nonspecific calpain inhibitors. Specific inhibitors for cathepsins B, K, S and L are available (CA-074, odanacatib, CLIK-060 and CLIK-148, respectively)[247,248]. Comparing the effects of these cathepsin inhibitors with those of calpain inhibitors such as ALLNal and MDL28170 may help to identify the responsible protease (or proteases) for various phenomena. Specifically targeting inhibitors by conjugating them with molecules with an affinity for particular sites or tissues — for example, carnitine (muscle)[127], taurine (neuron)[214] or pregabalin (brain)[215] — is another promising method.

At the molecular level, some differences in the substrate specificities of calpain-1 and calpain-2 have been recently revealed[98], and distinct biological functions of these calpains have also been reported[150,257–260]. Thus, differential inhibitions of calpain-1 and calpain-2 may improve the efficacy of some calpain-targeted drugs. One of the peptidyl α-ketoamide derivatives shows more than 100-fold selectivity for calpain-2 over calpain-1 (mCalp-I; Supplementary information S2 (table))[261,262]. Although a calpain-1-specific inhibitor has not yet been found (PD151746 shows only modest selectivity for calpain-1 over calpain-2, approximately 20-fold; see Supplementary information S2 (table)), the existence of mCalp-I indicates that such inhibitors are worth seeking.

can improve memory and synaptic function in mice overexpressing the amyloid precursor protein (APP), a model of AD[109]. This view was supported by findings that CAST deficiency induces the hyperphosphorylation of tau, somatodendritic atrophy, early lethality, augmented amyloid-β (Aβ) amyloidosis and elevated neuroinflammation in *APP*-overexpressing mice[109]. However, most of these phenotypes could not be reproduced using a second-generation mouse model of AD, which accumulates $A\beta_{42}$ without overexpressing *APP*; thus, the role for calpain in AD pathogenesis was challenged[20,110]. A recent study has shown that overexpression of *APP* and presenilin in mice causes calpain activation, indicating that preclinical studies should not unduly rely on overexpression paradigms[111]. Nevertheless, CAST deficiency accelerates Aβ amyloidosis and neuroinflammation in the second-generation mouse model, indicating that the calpain–CAST system may regulate the fundamental pathological processes of AD[20,110].

Calpain activity also appears to be an important player in other neurodegenerative diseases. For example, CAST overexpression reduces protein aggregation and synaptic impairment in mouse models of Parkinson disease (PD)[19]. In addition, the calpain-dependent cleavage of TDP43, a 43 kDa heterogeneous nuclear ribonucleoprotein that binds to the TAR DNA sequence and regulates transcription and RNA splicing, is an essential contributor to amyotrophic lateral sclerosis (ALS) aetiology[15]. Furthermore, CAST deficiency aggravates the pathology and symptoms of animal models of spinocerebellar ataxia type 3 (REF. 16).

Calpain inhibition may also be therapeutically beneficial in lissencephaly, a developmental brain disorder caused by defective neuronal migration[14]. Turnover of one of the responsible gene products, LIS1, is regulated by calpain proteolysis, and a heterozygous loss of *LIS1* results in insufficient amounts of LIS1 protein, causing defective intracellular microtubule network (homozygous loss results in lethality)[14]. Administration of calpain inhibitors to *Lis1*$^{+/-}$ mice (a model of lissencephaly) after disease onset significantly rescued defects in brain functions related to motor behaviour and glucose metabolism[14,17]. Calpain inhibitors tested in *Lis1*$^{+/-}$ mice include small interfering RNA (siRNA) targeted against *Capns1*, ALLNal (also known as calpain inhibitor I; an inhibitor of calpain-1 and calpain-2 and cysteine cathepsins) and SNJ1945 (a cell-permeable second-generation inhibitor of calpain-1 and calpain-2)[14,17].

Table 2 | **Diseases involving calpains and their therapeutic strategies**

Disease	Related calpain protein or gene (source)	Effect of activity	Calpain-related therapeutic candidates	Refs
Disease with a primary cause other than calpains				
Atherosclerosis	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors	25
Brain ischaemia	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors	24,44
Cardiovascular disorders	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors	23,39,114,116, 117,119
Cataracts	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors	92,134,136–139
Neurodegenerative disorders (e.g. Parkinson disease, amyotrophic lateral sclerosis, spinocerebellar ataxia type 3 and lissencephaly)	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors	14–17,19
Retinitis pigmentosa	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors	141,142
Cancers	Calpain-1, -2 CAPN3 and *CAPN9* (human, mouse)	Preventive, aggravating or causative	Calpain inhibitors and/or gene therapy	29,85,86,145, 147–159
Alzheimer disease	Calpain-1 and -2 (human, mouse)	May be aggravating or causative	Potentially calpain inhibitors	20,110
Muscular dystrophies	Calpain-1 and -2 (human, mouse)	May be aggravating or causative	Potentially calpain inhibitors	130
Disease model				
Cardiac scar	Calpain-1 and -2 (human, mouse)	Preventive	Potentially gene therapy	120
Dilated cardiomyopathy	Calpain-1 and -2 (human, mouse)	Preventive	Potentially gene therapy	121
Infectious disease				
Fungal infection (opportunistic infection)	PalB/Rim13 (fungus)	Aggravating or causative	Calpain inhibitors	181,182
Malaria	Calpain-1 and -2, *Pf*-calpain (human*, mouse*, parasite)	Aggravating or causative	Calpain inhibitors	41,178
Periodontitis	Tpr (bacteria)	Aggravating or causative	Calpain inhibitors	12
Trypanosomiasis and leishmaniasis (e.g. African sleeping sickness)	Calpain-1 and -2, ClpGM6 (human*, mouse*, parasite)	Aggravating or causative	Calpain inhibitors	180
Schistosomiasis	Smp-157500 (parasite)	Aggravating or causative	Vaccine (rSm-p80)	10,225
Calpainopathies				
Autosomal dominant neovascular inflammatory vitreoretinopathy	CAPN5 (human, mouse)	Aggravating or causative	Calpain inhibitors	34,167
Eosinophilic oesophagitis	CAPN14 (human, mouse)	Preventive	Potentially gene therapy	35,36
Gastric ulcer	CAPN8, CAPN9 (human, mouse)	Preventive	Potentially gene therapy	33
Limb-girdle muscular dystrophy type 2A	CAPN3 (human, mouse)	Preventive	Potentially gene therapy	32
Spastic paraplegia 76	CAPN1 (human, mouse)	Preventive	Potentially gene therapy	48
Type 2 diabetes	*CAPN10* (human, mouse)	Not definable	Unclear	251,252

Pf, *Plasmodium falciparum*; rSm-p80, recombinant Smp-157500 (carboxy-terminal 80 kDa fragment). *Calpains in host cells — that is, human or mouse — are used by the parasite.

Brain ischaemia. Brain ischaemia is caused by various insults, including cerebral infarction, haemorrhage, vasospasm and, sometimes, cardiac infarction. Brain ischaemia results in neuronal glucose starvation, ATP depletion and a rise in Ca^{2+} levels[44]. Transient cerebral ischaemia in gerbils is accompanied by calpain activation, which occurs in two phases: an immediate activation of calpain in hippocampal CA3 neurons followed by substantial calpain activation in CA1 neurons approximately 7 days later[112]. This process results in an overall delay in cell death of CA1 neurons, which can be blocked by administering the experimental calpain inhibitor ALLNal[24].

However, calpain is not easy to activate under physiological conditions. For example, almost lethal doses of kainic acid are required to achieve the forced activation of brain calpain, even in *Cast*$^{-/-}$ mice[105]. The observation that calpain is not widely activated in the brain supports the idea that if spatiotemporally restricted in the brain for a short period, a calpain-specific inhibitor would generate only minor side effects on physiological processes, at least in the experimental context.

Cardiovascular disorders. One of the pathological mechanisms underlying many acute cardiovascular disorders[8,113], including ischaemia–reperfusion and pressure-overload models, is thought to involve the calpain-mediated proteolysis of myocardial proteins[114,115]. Accordingly, calpain inhibitors are able to ameliorate symptoms of these disorders in animal models (TABLE 3). A protective effect of ALLNal, but not of a broad caspase inhibitor, on the myocardial injury and mitochondrial dysfunction caused by ischaemia–reperfusion revealed that calpain activation can result in cell death independent of caspases[116]. More specifically, mitochondrial calpain-1 was suggested to be involved in cardiac injury[117]; this injury could be prevented by the calpain inhibitor MDL28170, a cell-penetrating dipeptidyl aldehyde inhibitor that also acts on cysteine cathepsins[118]. MDL28170 is also called calpain inhibitor III (see Supplementary information S1,S2 (figure, table)).

Another calpain inhibitor, A-705253, a benzoylalanine-derived α-ketoamide inhibitor with improved water solubility, metabolic stability and cell permeability, exhibited protective effects on heart functions and global haemodynamics in a porcine myocardial ischaemia–reperfusion model[119]. Results from mice in which calpain activity was genetically inhibited by transgenic CAST expression or *Capns1* disruption also suggested that calpain is an aggravating factor in the chronic cardiac complications associated with type 1 diabetes or angiotensin II infusion[23,39]. Furthermore, the calpain inhibitors ALLMal, calpeptin and BDA-410 suppress atherosclerosis in *Ldlr*$^{-/-}$, *Apoe*$^{-/-}$ and angiotensin II-treated *Ldlr*$^{-/-}$ mice by blocking the proteolysis of vascular endothelial cadherin and/or spectrin[25,26] (TABLE 3).

Although calpain inhibition appears to be a promising approach for the treatment of several cardiovascular disorders, some adverse effects of calpain inhibition have been reported. For example, dilated cardiomyopathy and impaired scar healing are observed in transgenic CAST mice, in which endogenous calpain activity is reduced[59,120]. In addition, cardiac-specific *Capns1*$^{-/-}$ mice, although healthy under normal conditions, show cardiac dysfunction under pressure overload[121]. These studies suggest that calpain activation is required under some pathological conditions to overcome cardiotoxicity.

One possible mechanism by which calpain protects heart function is related to its role in membrane repair[89,122]. Meanwhile, studies using human blood cells and mouse models have indicated that calpain has opposing effects on inflammation and immune processes depending on the context, such as its localization[123]. In addition, an upregulation of CAPN2 was associated with the effect of a potential cardioprotective reagent, which is being considered for use in combination chemotherapeutic drug therapy[124]. Further clarification of the precise targets and timing of calpain activity will help us to evaluate the balance between the possible benefits and limitations of calpain inhibition.

Myopathies. In DMD, the activities of conventional calpains are upregulated due to a loss of Ca^{2+} homeostasis[2,113]. Several muscle proteins are substrates of calpains; thus, increased calpain activity is thought to exacerbate symptoms. In fact, studies using the dystrophin-deficient DMD mouse model, *mdx* mice, demonstrated that the increased calpain activation in necrotic muscle fibres was corrected in CAST overexpression in *mdx* mice, accompanied by the amelioration of the dystrophic phenotype[103,125]. Thus, proposed pharmacological approaches for ameliorating DMD have long included calpain inhibition. For example, the ability of inhibitors such as E-64d and leupeptin to delay muscle degeneration has been extensively studied[45,126] (see below). Promising results in model animals have led to further studies seeking to improve the bioavailability of these inhibitors in skeletal muscle tissues[127].

More recent studies using *mdx* mice[128] and a golden retriever DMD model[129], however, did not support the long-standing view that the pharmacological inhibition of calpains ameliorates DMD pathology. These studies showed that a decrease in calpain activity does not necessarily improve muscle functions, and also warned that chronic calpain inhibition by either inhibitor administration or *CAST* overexpression induces a compensatory upregulation of calpains. Thus, the outcomes of such treatments or conditions need to be carefully and consistently analysed[130].

Meanwhile, α-klotho, defects in which causes senescence, is specifically suppressed in the muscles of *mdx* mice after the clinical onset of muscular dystrophy, and transgenic α-klotho expression rescues the muscle atrophy phenotype in these mice[131]. The α-klotho protein modulates Ca^{2+} homeostasis, and *Kl*$^{-/-}$ mice exhibit overactivated calpain-1 and phenotypes reminiscent of ageing-related disorders. Such phenotypes include infertility, body weight loss, multiple organ atrophies, ectopic calcification and bone mineral density reduction[132]. These symptoms are ameliorated by the administration of the calpain inhibitor BDA-410 (REF. 133), implicating a role for α-klotho in regulating calpain activity (TABLE 3). Downregulation of α-klotho is also found in muscle biopsies from patients with DMD[131], suggesting that calpain inhibition might be more therapeutically effective if combined with another treatment that compensates for a deficiency in α-klotho function.

Ophthalmic diseases. Cataracts, a prominent age-related disease, result from the aggregation of crystallines, which is caused by oxidative damage and/or by over-proteolysis by calpains and other proteases[31]. Calpain-2 has a major role in human cataractogenesis by proteolysing the major lens proteins α- and β-crystallins[134], whereas variants of CAPN3, such as Lp82, and CAPN10 are also involved, especially in rodent models[135]. In transgenic mice expressing K6W mutant ubiquitin in the lens, the altered ubiquitin–proteasome system causes calpain hyperactivation, resulting in disease progression[136]. Thus, the development and application of calpain inhibitors for the treatment of cataracts has been an active topic in calpain research. *In vitro*, a preventive effect of calpain inhibitors against cataractogenesis in cultured rat lenses was first demonstrated for E-64 (REF. 137), and later for SJA6017 (REF. 92), a more potent calpain

Table 3 | **Selected *in vitro* and *in vivo* studies (since 2010) of calpain inhibition in disease**

Inhibitor or vaccine	Disease/ phenomenon	Model	Target*	Effects	Refs
ALLNal	Coxsackievirus B3 infection and replication	H9c2 cells	Calpains	• ↓ Virus titre • ↓ Autophagy	11
	Uterine implantation	Mice	Calpains (CAPN2)	• ↓ Implantation	246
	Lissencephaly	*Lis1*$^{+/-}$ mice	Calpains	• No change in LIS1 cleavage • ↓ Spectrin-α cleavage • ↓ Hyperexcitability (spontaneous and miniature excitatory postsynaptic current)	267
ALLNal, PD150606	Retinitis pigmentosa	Royal College of Surgeon's rats	Calpains	• ↓ Retinal cell apoptosis	268
ALLNal, calpeptin	Oestrogen-mediated cancer metastasis	Oestrogen and pure anti-oestrogen fulvestrant (ICI 182 780)-treated MCF-7 cells	CAPN1	• ↓ Cell–Matrigel adhesion	269
Calpeptin	Parkinson disease	MPTP-induced acute Parkinsonian mice	Calpains	• ↓ Glial activation • ↓ T cell infiltration • ↓ Neuronal death • ↓ Gait deficit	270
	Multiple sclerosis	EAE in Lewis rats	Calpains	• ↓ Gliosis • ↓ Loss of myelin • ↓ Axonal damage • ↓ Inflammation and microgliosis in optic nerve	271, 272
	Idiopathic inflammatory myopathies	IFNγ- or TNF-treated rat L6 myoblasts	Calpains	• ↓ MHC class I- and inflammation-related transcription factors • ↓ Apoptosis	273
	Idiopathic pulmonary fibrosis	Bleomycin-induced pulmonary fibrosis in mice	Calpains	• ↓ Lung fibrosis • ↓ Bleomycin-induced transcriptional upregulation of CAPN1, CAPN2, IL-6, TGFβ1, angiopoietin 1 and collagen type Iα1	274
MDL28170, calpeptin	Melanoma cell survival	Cisplatin-treated Me21 melanoma cells	Calpains	• ↓ Activation of caspases 3 and 7 • ↓ Cisplatin-induced apoptosis • ↑ Autophagy	160
MDL28170	Cardiac ischaemia–reperfusion	Mice	CAPN1	• ↓ Spectrin or junctophilin 2 cleavage • ↓ Damage on mitochondrial function	117, 275
	Chronic heart failure	Cardiomyocytes of dogs of chronic heart failure	Calpains	• ↓ Augmentation and altered kinetics of late Na^+ current	276
	Leishmaniasis	*In vitro* growth of promastigotes of *L. amazonensis*	*L. amazonensis* calpain‡	• ↑ Apoptosis (cell cycle arrest and DNA fragmentation)	173
	Chagas disease	*T. cruzi* epimastigotes infection *in vitro*	*T. cruzi* calpain‡	• ↓ Attachment to insect midgut • ↓ Differentiation • ↓ Viability	277
	Pre-eclampsia	BeWo cells	Calpains	• ↓ Apoptosis-inducing factor cleavage	278
	Spinal cord injury	Rats with spinal cord transection	Calpains	• ↓ Cleavage of α-subunit of voltage-gated Na^+ 1.6 channel • ↓ Persistent inward Na^+ current • ↓ Spasms	249
MDL28170, mCalp-I	LTP	Rats	CAPN1 and CAPN2	• ↓ LTP • ↓ SCOP degradation (MDL28170, but not mCalp-I) • ↓ PTEN degradation (mCalp-I)	261
AK295, calpastatin peptide (CAST)	Retinitis pigmentosa	C3H *rd1/rd1* mice	Calpains	• ↓ Photoreceptor cell death (AK295, short term; CAST, short and long term) • ↑ Photoreceptor cell death (AK295, long term)	279

Table 3 (cont.) | **Selected *in vitro* and *in vivo* studies (since 2010) of calpain inhibition in disease**

Inhibitor or vaccine	Disease/ phenomenon	Model	Target*	Effect	Refs
SNJ1945	Multiple sclerosis	EAE in mice	Calpains	• ↓ Paralysis, • ↓ T_H1 and T_H17 cell inflammatory responses • ↓ Induction of calpain	22
	Retinal ischaemia	Rats	Calpains	• ↑ Electrophysiological function of inner retinal layers • ↓ Loss of cone-ON bipolar and amacrine cells	280
	Lissencephaly	*Lis1*$^{+/-}$ mice	Calpains	• ↓ LIS1 cleavage • ↑ Corticogenesis, • ↑ Behavioural performance • ↑ Brain metabolism	17
	Cardiac ischaemia–reperfusion	Rats	Calpains	• ↓ Spectrin-α and SERCA2A cleavage • ↑ Left ventricular function	281
	Cortical impact traumatic brain injury	Mice	Calpains	• ↓ Spectrin-α cleavage	282
BDA-410	Machado–Joseph disease	Lentiviral expression of 72xGln-ataxin 3 in mice	Calpains	• ↓ Ataxin 3 cleavage • ↓ Cerebellar degeneration • ↓ Motor behavioural deficits	283
	Ageing-related syndromes	α-*Klotho*$^{-/-}$ mice`	CAPN1	• ↑ Reproductive ability • ↑ Body weight • ↓ Organ atrophy • ↓ Ectopic calcifications • ↓ Bone mineral density reduction • ↓ Pulmonary emphysema • ↓ Senile atrophy of skin	133
	Sickle cell disease	*Hbb*$^{single/single}$ SAD1 mice	CAPN1	• ↑ RBC morphology • ↓ RBC density • ↓ Hypoxia-induced RBC dehydration	220
	Abdominal aortic aneurysms and atherosclerosis	Hypercholesterolaemic *Ldl*$^{-/-}$ mice	CAPN1	• ↓ Spectrin-α cleavage • ↓ Abdominal aortic width • ↓ Atherosclerotic lesion	26
CYLA	Retinal ischaemia	Rats	Calpains	• ↑ Electrophysiological function of retina	218
A-705253 (BSF 419961)	Alzheimer disease	NMDAR-mediated neurodegeneration and Aβ-induced synaptic deficits in mice	Calpains	• ↓ Neuronal cell death • ↓ Caspase 3 activation • ↓ Deficits in synaptic transmission	284
	Alzheimer disease	Mice harbouring APP:K670M or APP:M671L, presenilin 1:M146V and tau:P301L mutations	Calpains	• ↓ Tau hyperphosphorylation • ↓ Proteolytic cleavage of CDK5 subunit p35 to p25	46
	Alcoholism	Cue-induced reinstatement of alcohol-seeking behaviour in post-dependent Wistar rats	Calpains	• ↓ Cue-induced reinstatement of alcohol-seeking behaviour • ↓ Alcohol (but not saccharine)-deprivation effects • No significant induction of NMDAR-mediated side effects (psychostimulant, cognition-impairing psychotomimetic effects)	285
Macrocyclic aldehyde (CAT811)	Cataracts	Lambs with inherited cortical cataracts	Calpains	• ↓ Lens cytoskeletal protein cleavage • ↓ Cataract development	139
NH_2-GRKKRRQRRR-PPQPDALKSRTLR-COOH (Tat-μCL), PD150606	Retinitis pigmentosa	Rats expressing rhodopsin:S334ter	CAPN1	• ↓ Retinal apoptosis	286
Dipeptidyl α,β-unsaturated ester	Malaria	*P. falciparum* parasitaemia in human erythrocytes or HeLa cells	Possibly *Pf*-calpain	• ↓ Parasite survival	287

Table 3 (cont.) | **Selected *in vitro* and *in vivo* studies (since 2010) of calpain inhibition in disease**

Inhibitor or vaccine	Disease/ phenomenon	Model	Target*	Effect	Refs
Hypervalent organotellurium compound (RF19)	Malaria	*P. falciparum* parasitaemia in human erythrocytes	Possibly *Pf*-calpain	• ↓ Parasite survival	219
rSm-p80 (recombinant protein)	Schistosomiasis	Baboon or hamster	Smp-157500 (*S. mansoni* calpain)	• ↓ Adult worms and eggs in tissues (hamster) • ↓ Egg excretion in faeces and urine (baboon) • ↑ Protective immune response	10, 225

Aβ, amyloid-β; APP, amyloid precursor protein; CDK5, cyclin-dependent kinase 5; EAE, experimental autoimmune encephalomyelitis; IFN, interferon; IL, interleukin; *L. amazonensis*, *Leishmania amazonensis*; LTP, long-term potentiation; MHC, major histocompatibility complex; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NMDAR, *N*-methyl-D-aspartate receptor; *P. falciparum*, *Plasmodium falciparum*; PTEN, phosphatase and tensin homologue; RBC, red blood cell; *S. mansoni*; *Schistosoma mansoni*; SCOP, suprachiasmatic nucleus circadian oscillatory protein; SERCA2A, sarcoplasmic reticulum Ca^{2+} ATPase; TGF, transforming growth factor; T_H, T helper; TNF, tumour necrosis factor; *Trypanosoma cruzi*, *T. cruzi*. *No specific calpain was targeted in the study unless specified. ‡Involvement of other cysteine peptidases has not been excluded.

inhibitor with good cell penetrability. Recently, a more specific macrocyclic inhibitor, CAT811 (ointment form), was tested on a sheep model of heritable cataract development; the inhibitor successfully slowed the development of the disease[138,139] (BOX 1; TABLE 3; Supplementary information S1 (figure)).

Calpain is also a therapeutic target for retinitis pigmentosa, which is caused by defects primarily in rhodopsins but also in various other genes, including those encoding carbonic anhydrase, pre-mRNA processing factors and phosphodiesterases[140] (TABLE 3). Retinitis pigmentosa is characterized by photoreceptor cell death, which results from calpain-mediated apoptosis induction[141], increased lysosomal membrane permeability[142] and/or downregulated heat shock protein 70 (HSP70)[143]. Thus, combining calpain inhibitors with inhibitors of cathepsin and caspase, as well as HSP70 inducers, could be an effective strategy for relieving the symptoms of retinitis pigmentosa.

Cancer. The calpain–CAST system has demonstrated opposing roles in cancer[30]. In many cases, the upregulation of CAPN1, CAPN2 and/or CAPNS1 is observed, whereas CAST is increased in others[27,144–146]. Activation of calpain-1 and calpain-2, and the proteolytic products of their substrates, have critical roles in cancer cell survival. For example, MYC-nick, a calpain-mediated cleavage product of the MYC proto-oncoprotein, promotes cytoskeletal remodelling, is upregulated in cancer cells and facilitates cell growth under hypoxia and nutrient deprivation, leading to malignancy[85,86]. Calpain–CAST is also involved in pathological angiogenesis, which is often observed in tumours. This angiogenesis is promoted by a loss of CAST, which amplifies the activity of calpain-2 (REF. 147). In addition, an accelerated calpain-mediated cleavage of receptors, such as the androgen receptor[148] and human epidermal growth factor receptor HER2 (also known as ERBB2)[149], contributes to cellular resistance to anticancer therapies. Calpain-1 and calpain-2 also regulate cellular machineries for drug efflux and hence reduce the efficacy of antitumour therapeutics such as tanespimycin, an HSP90 inhibitor derived from the antibiotic geldanamycin[29].

Recently, calpain-1 activity was shown to be important in the treatment for myelodysplastic syndrome (MDS)[150]. MDS is a haematopoietic stem cell disorder characterized by ineffective haematopoiesis and a tendency for acute myeloid leukaemia progression. Lenalidomide, a synthetic glutamic-acid-derived immunomodulatory drug, is used to treat MDS as well as multiple myeloma[151]. The sensitivity of myeloid cells to lenalidomide is dependent on calpain-1 but not calpain-2 activity, and suppression of CAST increases the susceptibility of MDS to lenalidomide[150]. These results indicate that in the treatment of MDS and possibly multiple myeloma, sufficient calpain-1 activity in the target myeloid cells is required for malignant cells to respond to treatment.

Unexpectedly, the unconventional CAPN3 (also known as p94) and CAPN9 (also known as nCL-4) have also been pathologically implicated in cancers. For example, CAPN3 is highly expressed in melanoma cell lines[152] and is downregulated in response to interferon-γ treatment[153]. CAPN3 is also found in bovine bladders with urothelial tumours[154]. Conversely, the CAPN3 isoforms hMp84 and hMp78 are upregulated in human melanoma cell lines treated with cisplatin to induce apoptosis, and downregulated in biopsies from human malignant melanocytic lesions[144]. Consistent with these observations, overexpressing hMp84, but not inactive hMp84:C42S, in melanoma results in cell death[155].

CAPN8 (also known as nCL-2) and CAPN9 are predominantly expressed in the gastrointestinal tract, and CAPN9 but not CAPN8 is suggested to be involved in the suppression of tumorigenesis[156–158]. Recently, downregulation of human CAPN9 but not CAPN8 was shown to correlate with unfavourable prognosis in patients with gastric cancer[159]. An expression study using stable human gastric cancer cell lines (MGC80-3 and MKN-45) showed that CAPN9 but not CAPN8 induced G1 cell cycle arrest and caspase-mediated apoptosis[159].

Other studies have suggested that calpain is required for apoptosis induced by the anticancer drug cisplatin in various cancer cell models[144,160], and that calpain inhibition is both beneficial and detrimental depending on the stage of tumour progression[145]. Thus, further studies are required to clarify whether calpains can both promote and suppress tumour growth and metastasis in different settings. More systematic studies examining, for example, whether the involvement of calpains depends on the cell type and/or calpain species, should clarify whether calpain-targeted strategies would be beneficial in cancer therapies.

Macrocyclic inhibitor
Macrocyclic structures introduced into linear peptides or compounds often improve potency by providing well-defined conformations, such as α-helices and β-strands in the case of peptides, thereby facilitating interactions with their targets, which include proteases. This strategy has been used to develop improved calpain inhibitors.

Calpainopathies

LGMD2A, the first calpainopathy. An important function of calpains was confirmed when CAPN3 was identified as the responsible agent for LGMD2A[32,93,161]. Prompted by this groundbreaking discovery, the term calpainopathy was explicitly defined as a disease condition that results from mutations in calpain genes. *CAPN3* is predominantly expressed in skeletal muscle and was the first non-ubiquitous member of the calpain superfamily to be reported[60]. LGMDs are characterized by their selective effect on the proximal muscles of limb girdles[162] (see also the Calpain 3 page of the Leiden Muscular Dystrophy website; see Further information). Worldwide, LGMD2A, an autosomal recessive LGMD, is the most frequent form of LGMD, and the rate of LGMD2A occurrence in some regions is much higher than average[162].

In LGMD2A, CAPN3 is devoid of protease function[93]. This finding is in stark contrast to the general concept that the pathology of muscular dystrophies is aggravated by a secondary overactivation of conventional calpains[130]. Moreover, studies using various mouse models of LGMD2A have revealed that CAPN3 also has a role as a regulatory component for Ca^{2+} release from sarcoplasmic reticulum in muscle cells in a manner independent of its protease activity[163,164]. Therefore, inhibiting CAPN3 would not be effective for LGMD2A and in fact could be detrimental (for an exception, see below).

Accordingly, the objectives of CAPN3-targeted therapy for LGMD2A are to restore or compensate for the loss of CAPN3 function[53]. To achieve these goals, essential questions that remain to be answered include how CAPN3 functions both as a protease and as a non-proteolytic modulator in muscle cells, what the *in vivo* targets of CAPN3 activity are, and in which biological pathways CAPN3 plays a part.

Other calpainopathies. *CAPN5* is responsible for autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV)[34,71]. *CAPN5* is expressed in most tissues, especially in the central nervous system[165,166]. So far, R243L, L244P and K250N have been identified as ADNIV-causative *CAPN5* mutations. Among these, R243L appears to be hyperactive, and transgenic mice overexpressing CAPN5:R243L show an ADNIV-like phenotype[167], whereas *Capn5*$^{-/-}$ mice exhibit no apparent phenotype[165]. Therefore, inhibiting CAPN5 (also known as hTRA3) in the retina is a possible therapeutic strategy for ADNIV; however, whether the Ca^{2+}-dependent proteolytic activity of CAPN5 is similar to those of conventional calpains needs to be explored. Alternatively, a mutant *CAPN5*-specific siRNA may be considered as a therapeutic approach.

CAPN14 has been identified as a genomic locus that is specifically associated with another calpainopathy, the allergic disorder eosinophilic oesophagitis[35,36]. *CAPN14* is predominately expressed in the oesophagus and is upregulated in eosinophilic oesophagitis or by IL-13 stimulation[35]. Intriguingly, an eosinophilic oesophagitis-associated risk single-nucleotide polymorphism (SNP) in *CAPN14* is intronic and decreases the expression level of *CAPN14*. The characterization of the protease activity of CAPN14 and its possible substrates suggests that downregulation of *CAPN14* compromises the cellular responses to IL-13 signals, whereas excess CAPN14 activity impairs epithelial barrier function[168]. The restoration of CAPN14 may therefore represent a logical therapeutic strategy, but its potential cytotoxicity must be monitored. Intriguingly, although the human *CAPN14* gene is localized to a genomic region that is syntenically conserved in mice and rats, mouse or rat *Capn14* cannot be found. This finding suggests that another calpain species in some rodents assumes the functions of CAPN14 and/or that the calpain-related aspect of the eosinophilic oesophagitis pathology does not present in these rodents.

More recently, another calpainopathy was reported: spastic paraplegia 76, a neurological disorder linked to *CAPN1* (REF. 48). Pathogenic mutations in *CAPN1* and other experimental evidence indicate that spastic paraplegia 76 is caused by a loss of calpain-1 function. Although no gross neurological abnormality is apparent in the constitutive *Capn1*$^{-/-}$ mouse, calpain-1 is known to have a neuroprotective role and to contribute to synaptic plasticity[18,169]. In addition, missense mutations in *CAPN1* are associated with spinocerebellar ataxia in dogs[170] and humans[171]. Taking this evidence together, it is strongly anticipated that calpain-1 will be a target not only for inhibition but also for genetic restoration therapeutic strategies.

Finally, with regard to additional potential calpainopathies, functional effects of SNPs in *CAPN8* and *CAPN9* merit attention. CAPN8 and CAPN9 are expressed in the gastrointestinal tract, especially in the mucus-secreting cells of the stomach, where they function as a heterodimer in complex with each other (called calpain-8/9 or G-calpain (G for gastric))[33]. *Capn8*$^{-/-}$, *Capn9*$^{-/-}$ and *Capn8*$^{C105S/C105S}$ mice are significantly more susceptible to gastric ulcers induced by ethanol stress than wild-type mice[33]. Importantly, several SNPs that inactivate CAPN8 or CAPN9 exist, suggesting that a susceptibility to gastric ulcers can be predicted by these SNPs. Therefore, strategies that compensate for and/or activate calpain-8/9, depending on the effect of the SNP, would be logical directions for treatment.

Infectious diseases

Parasitic diseases. Calpains expressed in parasites play crucial parts in their pathogenicity; thus, calpains represent promising anti-disease targets. Notably, calpains are involved in the pathogenicity of trypanosomiasis[172], leishmaniasis[172,173] and schistosomiasis[174], which are among the neglected tropical diseases recognized by the World Health Organization[175].

Malaria caused by *Plasmodium falciparum* is one of the most serious parasitic diseases[176]. *P. falciparum* uses aspartyl and cysteine proteases to degrade host haemoglobins and to survive as a parasite[177], and *Pf*-calpain is essential for the life cycle of the parasite in cells[178], suggesting that these proteases are promising drug targets. Although host calpain-1 is reported to be required for the efficient egress of *P. falciparum*[41], the invasion and growth of parasites in erythrocytes from wild-type and *Capn1*$^{-/-}$ mice show no significant difference[179].

Neglected tropical diseases
Among various parasitic and infectious diseases, 17 diseases have been recognized by the World Health Organization as targets for which control would promote an exodus from poverty somewhere in the world.

Table 4 | **Selected calpain-related therapeutic agents**

Compound (alternative names)	Company or institute	Disease	Phase	Details	Refs
ABT-957	AbbVie	Alzheimer disease	Phase I, terminated	Randomized, multiple-dose-escalation safety, tolerability and pharmacokinetics study	288
ABT-957	AbbVie	Alzheimer disease	Phase I, terminated	Randomized, multiple-dose-escalation safety and efficacy study	289
Olesoxime	Trophos	Relapsing-remitting multiple sclerosis	Phase I, completed	Randomized, single-dose safety study	290
Olesoxime	F. Hoffmann-La Roche	Spinal muscular atrophy	Phase II/III, in progress	Single-dose safety, tolerability and efficacy study	291
Olesoxime	Trophos	Amyotrophic lateral sclerosis	Phase II/III, completed	Multicentre, open-label safety extension study	292,293
Cyclosporine A	E. Hall	Traumatic brain injury	Phase I/II, in progress	Randomized, pharmacokinetics and pharmacodynamics study; evaluated by calpain-mediated cytoskeletal breakdown products	294
E-64d (EST, Estate, loxistatin and rexostatine)	Taisho Pharmaceutical	Muscular dystrophy	Phase III, discontinued	Effective for protease inhibition to delay muscle protein degradation and disease progression	45
AK-295 (CX295)	Alkermes	• Stroke • Cataract	Preclinical, discontinued	Systemic inhibition of calpain to suppress side effects of cancer therapy on sensory neurons	295
C-101 (myodur and CLA)	CepTor	Muscular dystrophy	Preclinical, discontinued	Increased myofibre diameter in *mdx* mice and low toxicity for rats	127
CYLA	The State University of New York	• Multiple sclerosis • Retinal ischaemia	Preclinical	Capable of crossing the blood–brain barrier through taurine transporter and effective for retinal ischaemia-associated lesion	217,218
C-201 (neurodur and CLA)	CepTor	• EAE • Acoustic trauma	Preclinical	Aldehyde form of CYLA; effective for slowing EAE progression in mice and treating acoustic trauma in chinchillas	214
CEP-3453	University of Pennsylvania, Teva	Ischaemic stroke	Preclinical	Neuroprotective 22 h post-ischaemia treatment in a rat model	296
CEP-4143	Cephalon	Spinal cord injuries	Preclinical, discontinued	Pre-injury treatment exhibited neuroprotection in rat model	297
Calpeptin (IPSI-001)	Medical University of South Carolina	Multiple sclerosis	Preclinical	Reduction of demyelination and axonal damage to treat multiple sclerosis-associated optic neuritis	272
Calpeptin (IPSI-001)	Washington University School of Medicine	Wolfram syndrome	Preclinical	One of disease-responsible gene products, WFS2, downregulates CAPNS1 via the ubiquitin–proteasome system	298
A-705239 (BSF 409425)	Abbott	• Acute myocardial infarction • Cerebrovascular diseases	Preclinical	Respiratory functions of mitochondria from the heart challenged by ischaemia–reperfusion was preserved	199,200
A-705253 (CAL 9961 and BSF 419961)	Christian-Albrechts University of Kiel	Acute myocardial infarction	Preclinical	Pre-infarction administration inhibited calpain and cardiac hypertrophy	299
Ala-1.0 (leupeptin linked to pregabalin)	City University of New York, Institute of Basic Research in Developmental Disabilities, The State University of New York	• Stroke • Alzheimer disease • Muscular dystrophy	Preclinical	Intraperitoneal administration immediately after controlled cortical impact decreased neurodegeneration in a rat model	216
rAAV2/1-CAPN3	French National Center for Scientific Research (CNRS)	Muscular dystrophy	Preclinical	Intramuscular delivery of *CAPN3* gene corrected dystrophic features of LGMD2A model mice	300
• Sm-p80-pcDNA3 • Sm-p80-VR1020	Texas Tech University Health Sciences Center	Schistosomiasis	Preclinical	Longevity of production of Sm-p80-specific antibodies (1–8 years) was demonstrated	226,301
rSm-p80	Texas Tech University Health Sciences Center	Schistosomiasis	Preclinical	Effect of vaccination to acute and chronic infection was shown	225

EAE, experimental autoimmune encephalomyelitis; LGMD2A, limb-girdle muscular dystrophy type 2A.

Another parasite spread by insects is *Trypanosoma*, which causes African sleeping sickness (*Trypanosoma brucei* transmitted by *Glossina* spp. (tsetse fly)), Chagas disease (*Trypanosoma cruzi* transmitted by *Reduviidae* spp. (assassin fly)), kala-azar and Oriental sore (*Leishmania donovani* and *Leishmania major* transmitted by *Phlebotominae* spp. (sand fly)), as well as other diseases[172]. Surprisingly, these parasites have 18–27 calpain genes[42], one of which, ClpGM6, is involved in the morphology determination of *T. brucei*[180]. ClpGM6 is an 820 kDa protein containing three copies of the CysPc and CBSW domains, and >70 repeats of the GM6 motif (a 68 amino acid residue unit) (FIG. 1). It is still unknown whether trypanosomal calpains function as proteases because most of them lack the conserved active site cysteine residue[42].

The blood flukes *Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium* are transmitted by *Planorbidae* spp. (planorbid or ramshorn snails) and cause schistosomiasis, which can include hepatosplenic inflammation, liver fibrosis and bladder cancer[174]. *S. mansoni* harbours seven genes that encode calpains, four of which are classical calpains and one is a *CAPN7* homologue. At least one of the classical calpains, Smp-157500 (FIG. 1), may be essential for the pathogenicity of this parasite given that it is an effective target for vaccines[10] (TABLES 3,4 and see below). The physiological functions of these calpains, however, are still elusive[50].

Fungal and bacterial infections. Of the 449 whole-genome-sequenced fungus genera, 431 have one or more calpain-encoding genes; important exceptions include *Schizosaccharomyces*, *Encephalitozoon* and *Pneumocystis* (see MycoCosm of the US Department of Energy's Joint Genome Institute Genome Portal; Further information). Candidiasis, cryptococcosis, aspergillosis, coccidioidomycosis and trichophytia are caused by infection with the fungi or yeasts *Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., *Coccidioides* spp. and *Trichophyton* spp., respectively, of various human tissues, such as the lung, mouth and skin[43]. These infections cause serious problems, especially in immunocompromised individuals, such as HIV-infected or organ transplant patients.

To infect human tissues, the pathogens described above adapt to the environmental pH by a mechanism that uses their own calpains, PalB/Rim13 (REFS 181,182). *RIM13* is the only *Saccharomyces cerevisiae* gene that encodes a calpain[183], and *palB* is one of the calpain genes of *Aspergillus nidulans*[184]; they are both orthologues of human *CAPN7*. The involvement of Rim13 and PalB in alkaline adaptation signalling was discovered in *A. nidulans* and *S. cerevisiae*, followed by elucidation of the detailed process[54]. A pH sensor (consisting of Rim21/Dfg16/PalH (7 spanners), Rim8/PalF (arrestin-like) and Rim9/PalI (chaperone)) transduces an alkaline signal to a proteolytic complex with the aid of ESCRT (endosomal sorting complex required for transport)-I, II and III. In this process, Rim20/PalA and Ygr122w/PalC are induced to act as a scaffold for the proteolytic activation of Rim101/PacC (transcription factor) by Rim13/PalB[54]. Thus, this signalling is called the Rim101 pathway.

Among the few bacterial calpains[52], Tpr of *Porphyromonas gingivalis*, which causes periodontitis, is required for the survival and infection ability of the bacteria, and was shown to be a Ca^{2+}-dependent cysteine protease with both autolytic and substrate (for example, fibrinogen) proteolysing activities[12].

Strategies for therapeutics

As discussed above, there are three types of calpain-related human disorders: those exacerbated by human calpain activities (type 1); those caused by pathogenic microorganisms that use the host's and/or their own calpains for infection and survival (type 2); and those caused by calpain gene deficiencies (type 3). For most of the type 1 and some of the type 2 disorders, inhibitors for the conventional calpains are the first therapeutic choice. Some microorganisms in type 2 disorders can infect or survive using their own calpains, which may therefore represent ideal specific targets. For type 3 disorders with defective calpains, gene therapy to restore calpain activity should be considered as a potential therapeutic approach. In addition, some type 3 disorders result in hyperactivation of calpains due to a gain-of-function mutation[167], for which the inhibition or reduction of calpain activity would be a logical therapeutic strategy.

Inhibiting conventional calpain activities. First-generation calpain inhibitors include leupeptin and E-64, originating from actinomycete and fungus, respectively, and their derivatives. These inhibitors exhibit little specificity for calpains and show broad inhibitory activity against not only clan CA proteases[118] but also matrix metalloproteinase 2 (REF. 185) (BOX 1; TABLE 3; Supplementary information S1,S2 (figure, table)). Nevertheless, leupeptin and its modified versions have been studied extensively and evaluated in pilot clinical trials[186]. One of these products, C-101 (also known as myodur; see below), although discontinued as a clinical candidate, represents an important approach to effectively deliver a drug to muscle cells by taking advantage of a receptor–ligand interaction[127] (see below) (Supplementary information S1 (figure)). Meanwhile, E-64d was shown to prolong the lifespan of a dystrophic hamster, UM-X7, and was used in clinical trials up to phase III in the 1980s[45]. Unfortunately, the results were inconclusive, and the trials were discontinued in 1992 (REF. 187). Several attempts to improve the specificity of these molecules resulted in second-generation inhibitors such as SJA6017 (REF. 92) and PD150606 (REF. 76). However, these agents were still not sufficiently specific to distinguish calpains from other proteases. Although endogenous CAST is currently the only absolutely specific inhibitor for classical calpains, strategies to improve inhibition are emerging (BOX 1).

Indeed, advances in structural biology have enabled logical structure design, which has led to a group of promising third-generation inhibitors with fixed secondary structures. Although proteases are known to generally recognize β-strands[188], most of the small-molecule protease inhibitors are conformationally flexible. This flexibility induces cooperative effects between the protease and the inhibitor (so-called induced fit[189]), resulting in unexpected enzyme–inhibitor interactions

at adjacent or even remote locations from the active site and, therefore, a loss of inhibitory specificity. This effect can be avoided by restricting the secondary structure of the inhibitor molecule. Among several attempts to achieve this goal, stable β-strands were achieved by the macrocyclization of inhibitor peptides[138,190,191].

Notably, although CAST is an intrinsically unstructured protein, the 3D structure of a calpain-2–CAST complex shows that α-helix and β-turn structures in CAST subdomain B induced by its binding to the active site of calpain-2 have essential roles in its specific inhibition. Thus, peptides made to mimic these structures by crosslinkers[192] or by cyclization[188] are inherently specific to calpains (with inhibition constant (K_i) values of 10–20 μM) (Supplementary information S1 (figure)).

In addition, an alternative approach is provided by a calpain-derived inhibitory peptide for mitochondrial calpain-1 that specifically blocks calpains[193]. Mitochondrial calpain-1 is associated with the molecular chaperone ERp57 via the CBSW domain of CAPN1 (REF. 194). Because inhibiting ERp57 destabilizes mitochondrial calpain-1 (REF. 194), synthetic peptides were screened for their ability to competitively inhibit the ERp57–CBSW interaction. This screen identified PDALKSRTLR, an oligopeptide corresponding to a linker region between the PC2 and CBSW domains of CAPN1 that inhibited mitochondrial but not cytosolic calpain-1 (median inhibitory concentration (IC_{50}) of 112 nM)[194]. A form of this peptide N-terminally conjugated with the cell-penetrating HIV-1 Tat sequence (GRKKRRQRRRPPQ) prevented photoreceptor cell death in retinal dystrophic rats (TABLE 3), with no inhibition of cathepsin L, papain or proteasomes[194]. This strategy is ideal in its specificity, and may be applicable to other calpains: for example, for the development of a peptide that interferes with the PEF(L) and PEF(S) interaction of conventional calpains.

As described above, the development of allosteric inhibitors of calpains is another important area for improving specificity. For this endeavour, elucidation of the precise inhibitory mechanisms of α-mercaptoacrylic acid derivatives, such as PD150606, is urgently required. Intriguingly, the dimerization of PD150606 and its derivatives via disulfide formation significantly increases their calpain inhibitory activity (for example, the IC_{50} values of PD150606 and its dimer are 5.0 μM and 7.5 nM, respectively)[195]. The dimerized molecules bind to the PEF domain similarly to PD150606 but more stably[195], although their inhibitory mechanisms are unclear. These studies have opened up a new direction for calpain-specific inhibitor development, in which the inhibitors are directed to regions other than the active site.

As a promising example, a novel allosteric site and its small-molecule inhibitor, NSC13345, for cathepsin K, a cysteine cathepsin with 3D structural similarity to the calpain CysPc domain, were discovered by computational methods[196]. NSC13345 inhibits cathepsin K by binding to a relatively flat side surface of the β-strand-rich region that harbours the active site histidine and asparagine residues, corresponding to the PC2 domain of calpains[196]. Therefore, similar methods should be applicable to finding calpain-specific allosteric sites and their docking molecules. Further information about the recent progress on calpain inhibitors can be found in other detailed and comprehensive reviews[3,197,198].

Preclinical and clinical agents in development. Calpain inhibitors are continuing to be developed therapeutically, and some of them are currently being tested in clinical trials (TABLE 4). Two structurally related α-ketoamide calpain inhibitors, A-705239 (also known as BSF 409425) and A-705253, show promise because of their improved water solubility, cell permeability and metabolic stability over other inhibitors, although they have some limitations with regard to specificity (Supplementary information S1,S2 (figure, table))[199]. Administering A-705239 after an induced traumatic brain injury in rats rescued brain cells, proving the biological efficacy of the drug[199]. In another disease model, acute myocardial infarction induced by ischaemia–reperfusion in a rabbit heart, the infarct size was reduced and the respiratory function of mitochondria was preserved when A-705239 was included throughout the procedure[200]. An improved derivative of A-705239 developed by AbbVie, ABT-957, had been in the first sets of clinical trials as a treatment for cognitive disorders until recently[198,201–203] (TABLE 4). The exact structure of ABT-957 has not yet been disclosed.

A neuroprotective cholesterol derivative, olesoxime (Supplementary information S1 (figure)), which has been subjected to clinical trials as a therapeutic reagent for motor neuron diseases such as ALS[204], spinal muscular atrophy (SMA)[205,206] and relapsing-remitting multiple sclerosis[207], was recently shown to suppress calpain activity in a rat model of Huntington disease[208,209]. The precise mode of action of olesoxime is currently elusive, but as it binds to outer mitochondrial membrane proteins and inhibits the efflux of apoptotic factors under neurotoxic or cytotoxic conditions *in vitro*[210], calpains are probably not its direct target. The safety and suitability of olesoxime for oral delivery have been demonstrated in clinical trials[206,211] (TABLE 4). A beneficial clinical outcome, such as a retardation of disease progression, was observed in a SMA trial focusing on the early phase of disease onset[205], but not in an ALS trial assessing survival at the end stage of the disease[211]. These results suggest that the neuroprotective effect of olesoxime depends on the timing of its administration during disease progression. Although the calpain activity in olesoxime-treated neurodegenerative diseases other than Huntington disease awaits investigation, it is possible that one of the main neuroprotective actions of olesoxime depends on calpain suppression. Notably, survival of motor neuron (SMN) protein, a gene product responsible for SMA, is a calpain substrate[212], and calpain upregulation has been reported in an ALS mouse model[213], suggesting that other calpain inhibitors may also be effective for treating these diseases as well as Huntington disease.

In parallel with the efforts to refine the core structure of calpain inhibitors, the covalent attachment of a tag motif to an inhibitor molecule that causes it to accumulate in a region of interest, such as muscle cells, has been examined[127,214–216]. Among these compounds, C-101 is a modified leupeptin linked to carnitine, which is efficiently targeted to skeletal muscle tissue, where the

Intrinsically unstructured protein
A protein that does not possess a fixed or stable 3D structure (also called an 'intrinsically disordered protein'). Some of these proteins remain unstructured even in their functional state, whereas others adopt a fixed structure after binding to another protein. For example, calpastatin has tandemly repeated calpain inhibitory sequences, neither of which assumes a defined structure unless calpastatin is bound to calpain.

carnitine receptor OCTN2 (also known as SLC22A5) is expressed[127]. At the preclinical level, C-101 increased the fibre diameter in the skeletal muscle of *mdx* mice, indicating its promise as a therapeutic reagent for muscular dystrophies[127]. However, the preclinical trial for this product was discontinued, and other variants that have been examined at the preclinical level await further evaluation. These inhibitors include C-201 (also known as neurodur)[214], GABAdur[215] and Ala-1.0 (REF. 216), which are attached to taurine or the anti-epileptic drug pregabalin and are designed to elicit neuroprotection by limiting calpain activation (Supplementary information S1 (figure)). CYLA is a diethyl acetal of C-201 that is converted to an active form after undergoing hydrolysis *in vivo*. Delivery of CYLA to the brain and prevention of axon injury was demonstrated in a mouse model of multiple sclerosis[217]. CYLA is also reported to prevent retinal cell degeneration in a rat model of retinal ischaemia[218].

Targeting calpain pathways in infectious diseases

Malaria and sickle cell disease. As the malaria-causing *Plasmodium* parasite requires proteases, including *Pf*-calpain, for its survival, these proteases are promising drug targets. Conventional calpain inhibitors also inhibit *Pf*-calpain: ALLNal and ALLMal suppress the erythrocyte invasion of *P. falciparum*[40], and BDA-410 blocks parasite growth *in vitro* and *in vivo*[9]. In addition, hypervalent organotellurium compounds inhibit the *Pf*-calpain-like activity[219] (TABLE 3). These potential drug candidates should be tested for their specificity using recombinant *Pf*-calpain[70].

Sickle cells disease (SCD) is caused by pathogenic mutations of the β-globin gene (*HBB*) and is accompanied by malaria resistance. Dense sickle cells are a hallmark of human SCD and show reduced levels of CAST[220]. Notably, the oral administration of BDA-410 in a mouse model of SCD (SAD mice) ameliorates sickle cell density and hypoxia-induced erythrocyte dehydration[220]. The proposed molecular mechanisms of sickle cell deficiency include the perturbation of PRX2 (also known as calpromotin), which protects against oxidative stress, and the upregulation of a Ca^{2+}-activated K^+ channel (Gardos channel) by overactivated calpain-1 (REF. 220). Consistent with this scenario, Gardos channel activity is suppressed in *Capn1*$^{-/-}$ mouse erythrocytes[221]. However, the *Capn1*$^{-/-}$ mouse erythrocytes become deformed upon Ca^{2+}-ionophore-induced echinocyte formation[221]. Thus, further investigations of the effects of calpain-1 inhibition on erythrocytes need to be conducted before applying calpain inhibitors to treat SCD.

Trypanosomiasis and leishmaniasis. MDL28170 shows trypanocidal effects without significant toxicity to the host cells[173] (TABLE 3). Treating *Leishmania amazonensis* promastigotes with 30 μM (double the IC_{50} dose) of MDL28170 efficiently suppresses their growth and viability, and induces an apoptosis-like cell morphology accompanied by cell cycle arrest and DNA fragmentation[173]. ALLNal, however, suppresses the apoptosis-like cell death of *L. donovani* induced by miltefosine (another trypanocidal agent), and E-64 has no effect[222]. These studies collectively suggest that the target of MDL28170 is unlikely to be calpain activity, but that *Trypanosomal* calpain-like molecules, such as CAP5.5 (*Tb*CALP1; FIG. 1), that lack protease activity may act as cytoskeletal modulators[180,223]. Although there is evidence to suggest that trypanosomal calpains would be good drug targets, inhibitors such as MDL28170 also act on host calpains, and detailed studies are needed to determine how the pseudo-proteolytic trypanosomal calpains function and how calpain inhibitors act against them.

Schistosomiasis. There is currently no vaccine for schistosomiasis for human use. However, one of the leading candidate target molecules for a schistosomiasis vaccine is Sm-p80, a C-terminal portion of the *S. mansoni* calpain Smp-157500 (FIG. 1). Smp-157500 is exposed on the membrane surface and has an important role in the surface membrane renewal and recycling of the parasite to evade the host immune response[10,224]. Although the precise function of Smp-157500 is currently elusive, baboon vaccination data for Sm-p80 are promising[10,225] (TABLES 3,4). Baboons chronically infected with *S. mansoni* were treated with a recombinant Sm-p80 protein (rSm-p80) or an Sm-p80-expression DNA vector plasmid along with adjuvants (Toll-like receptor 4 agonist-based glucopyranosyl lipid (GLA-SE) or aluminium hydroxide (alum))[10]. Among several combinations, rSm-p80 plus GLA-SE was the most effective, resulting in the production of immunoglobulin A (IgA) in addition to IgG and IgM, a 36% reduction in the number of worms, and 54% and 33% reductions in the amounts of tissue and faecal eggs, respectively[10]. The same strategy was also effective in baboons and hamsters infected with *S. haematobium*, the Sm-p80 amino acid sequence of which is 95% identical to that of *S. mansoni*[225]. Surprisingly, the elicited Sm-p80-specific IgG in vaccinated baboons was still detected 5–8 years after immunization[226]. These studies support the testing of an Sm-p80 vaccine in human clinical trials.

Diseases caused by fungi, yeasts and bacteria. Inhibitors specific for calpains in fungi, yeast and bacteria are promising therapeutics for diseases caused by these pathogens. For example, *C. albicans* infects and lives on mucosal surfaces of the human gastrointestinal and genitourinary tracts by expressing several genes for alkaline adaptation, such as superoxide dismutase 4 (*SOD4*) and *SOD5* and aspartyl proteases (*SAP5* and *SAP6*), causing candidiasis[13]. Deletion of *RIM101* effectively disrupted this infection by downregulating Rim101-induced genes[13]. Similarly, *Cryptococcus neoformans* uses the Rim101 pathway for infection, and causes life-threatening meningitis in immunocompromised humans[182]. Rim13 proteins of these yeasts are homologues of PalB and CAPN7 (also known as PalBH), which constitute the most divergent calpain subfamily, and have further diverged CysPc domains even compared with that of human CAPN7 (~20% identity)[50]. Thus, it is possible to design inhibitors specific to these yeast Rim13 proteins without inhibitory activity to human CAPN7, which is thought to be essential for human cellular functions[227]. However, so far, no inhibitor has been

Box 2 | **Activators of calpains**

Many enzymes have efficient activators, such as phorbol esters and diacylglycerol for protein kinase C, AMP for AMPK, and the small GTPase RAC for NADPH oxygenase. Calpain activators could be used, for example, to increase calpain activity in attenuated activity-type calpainopathies and in some cardiovascular disorders requiring a transient activation of calpains. The conventional calpains are activated by Ca^{2+}, Mg^{2+} and phospholipids, which are common activators for various enzymes, and hence could not be used as a specific activator. Although activator macromolecules for calpains (UK114, acyl CoA-binding protein, DNA and calpastatin fragments, among others) have been reported, none of them has survived further analysis. Why is a calpain activator so elusive?

One possible reason is that calpain activity has to be strictly suppressed in the cell. In fact, conventional calpains proteolyse more than 40% of the peptide bonds of most polypeptides when exhaustively reacted *in vitro* (F. Shinkai-Ouchi, Y.O., T.C.S. and H.S., unpublished observations). Thus, there are multiple safety features that regulate the activity of conventional calpains, such as the very high [Ca^{2+}] requirement for full activity and the lack of an active site conformation in the absence of Ca^{2+}. In addition, the deep active site cleft is inaccessible for many structured polypeptides, and the specific inhibitor calpastatin is expressed in excess amounts in most cells. However, the recently reported intermolecular complementation phenomenon of CAPN3 (REF. 232) provides a potential new direction for the development of a calpain activator, in that some parts of calpain itself might function as an activator.

designed, and no 3D structures have been determined for Rim13. These topics are challenging but urgent for future study. Conventional calpain inhibitors may also be effective in controlling these diseases. For example, *Shigella flexneri*, a dysentery-causing bacteria, does not have its own calpain but escapes from the host immunity by activating conventional calpains[228].

Restoring intrinsic functions of calpains

LGMD2A is the most thoroughly studied human calpainopathy confirmed to be caused by the genetic loss-of-function of a calpain gene (*CAPN3*), and is therefore the leading example for which the complementation of calpain activity would be an appropriate therapy. Among other calpainopathies reported so far, those caused by defects in *CAPN1* (REF. 48), *CAPN8* (REF. 33), *CAPN9* (REF. 33) and *CAPN14* (REFS 35,36) would also require restoration by complementation.

There are several challenges facing the diagnosis and treatment of LGMD2A. A definitive diagnosis of LGMD2A requires the identification of pathogenic mutations in *CAPN3*, which encompasses more than 60 kb, and this test is time consuming and costly. A frequently used alternative is to check the CAPN3 protein by western blot analysis. However, a normal level of CAPN3 is found in ~30% of patients with LGMD2A[162], and clinicians need to be aware of this fact when using this method. Analysing the Na^+-dependent autolytic activity, a unique feature of CAPN3 (REF. 229), to assess the activity of CAPN3 would be a better diagnostic method. Furthermore, the nature of the protease activity of CAPN3 remains unclear: that is, where it is activated, in what forms and under what circumstances.

Gene therapy. Gene therapy is currently the most practical approach for calpain replacement because the sizes of calpains (~100 kDa) are appropriate for viral vectors, and no activators for these calpains are currently available (BOX 2).

In the course of developing strategies for correcting genetically defective *CAPN3* by gene transfer, it was revealed that skeletal but not heart muscle has the capacity to regulate CAPN3, and that freely active CAPN3 can be detrimental to tissues in which it is accidently expressed[47,59]. To address this issue, skeletal-muscle-specific promoters and/or cardiac-specific gene suppression by microRNAs are effective strategies that do not decrease the expression level of the transferred *CAPN3* gene in skeletal muscle[47] (TABLE 4).

Therapeutic strategies to correct causative gene mutations by gene editing techniques combined with the use of patient-derived induced pluripotent stem cells are also on the horizon[230,231]. Therapies for calpainopathies, depending on the nature of the affected tissues and the mutation, could greatly benefit from such advancements. More than one-third of the cases of LGMD2A are caused by missense mutations[162] (see also Further information), which are appropriate for gene editing therapy.

Intermolecular complementation of CAPN3. An alternative approach to correct CAPN3 activity is suggested by an unusual activation ability of CAPN3. CAPN3 undergoes rapid autolysis; however, it was recently discovered that the protease activity, including its autolytic activity, can be restored through the intermolecular complementation (iMOC) of autolysed fragments[232]. Such iMOC had only previously been described for some proteases of viruses, the self-remodelling of which is involved in efficient infectivity[233]. The iMOC of CAPN3 is thought to be a mechanism for regulating its activity and localization[232]. Therefore, in some specific cases, it might be possible to correct dysfunctional CAPN3 mutants by delivering a defined region of CAPN3, which is not in itself active because it is not the whole molecule[232,234].

Inhibition of LGMD2A mutant CAPN3. An *in vitro* study indicated that some LGMD2A mutant CAPN3 proteins, such as those mutated in the PEF domain, have accelerated autolytic activity, so they cannot function owing to their rapid autodegradation[93,235]. It may therefore be possible to use partial inhibitors that slow down this autolysis but do not competitively inhibit the protease activity. For example, PD150606 binds to PEF domains[76], although its primary site of action is the CysPc not the PEF domain (see above)[79]. One of the amino acid residues, F226 of PEF(S), that contacts PD150606 or its derivatives, is conserved in the PEF(L) of CAPN3, a region of LGMD2A pathogenic mutation (F779I)[236] (see also Further information). Therefore, PD150606 and/or its derivatives may alter the activity of PEF-domain-mutated CAPN3 proteins with moderate efficiency. In addition to LGMD2A caused by hyper-autodegrading *CAPN3* mutations, some other conditions may be ameliorated by inhibiting CAPN3. For example, tibial muscular dystrophy is primarily caused by mutations in the titin (*TTN*) gene, which in turn dysregulate CAPN3 (REF. 237). Cardiomyopathy phenotypes collaterally caused by misexpression of *CAPN3* in heart muscle, which is a problem in applying gene therapy for LGMD2A[47], could also be

Intermolecular complementation (iMOC). A phenomenon in which single-polypeptide-derived fragments, none of which are capable of expressing the activity of the original protein, reconstitute the original activity through spontaneous and noncovalent interaction under physiological conditions. In this process, the amino acids essential for the activity are provided by different fragments.

prevented by inhibiting CAPN3 in the heart (TABLE 4). In these cases, the development of CAPN3-specific inhibitors would be helpful.

Increasing muscle mass. A different approach to treat LGMD2A, as well as other muscular dystrophies, is to promote muscle development and regeneration, as is the aim of anti-myostatin treatment[238,239]. In this respect, CAPN6 (also known as CANPX), the only naturally inactive human calpain (FIG. 1), represents a unique factor regulating skeletal muscle mass[240]. *Capn6* gene-disrupted mice exhibit skeletal muscle hypergenesis, and mice with myostatin (*Mstn*) gene disruption exhibit increased muscle mass[240,241]. Therefore, counteracting CAPN6 and/or its downstream molecules, which largely remain elusive, by specific antibodies or siRNA may ameliorate the phenotypes of muscular dystrophies. The effect of CAPN6 inhibition on muscular dystrophies, which may vary depending on the genes responsible for disease, the model animal and/or the methodology of inhibition, as has been reported for myostatin[242,243], warrants further investigation.

Challenges in calpain-targeted therapies

The conventional calpains are expressed in almost all cells. However, because their physiological roles are generally auxiliary, their inhibition does not cause serious problems under normal conditions. This finding explains the rationale for using conventional calpain inhibitors to treat various diseases that are aggravated by calpain activity. However, calpain activity is required for some processes that involve the orchestration of multiple molecular functions. Therefore, the safety of chronically inhibiting calpain activity must be considered. Indeed, detrimental effects of calpain inhibition on cardiomyocytes[59,121], the immune system[120,123,244,245], uterine implantation[246] and cancer suppression[150,155–159] have already been reported (TABLE 3). The use of calpain inhibitors and potential side effects must also be studied under various conditions: for example, under a perturbed immune system due to disease, the environment and/or ageing.

Another issue is that in some studies of acute disorders, such as cardiovascular disorders, the calpain inhibitors are administered *ex ante*, which does not happen in actual therapy. The effects of calpain inhibition initiated after disease onset would be of more practical value. Except for preventing the effects of infectious diseases or the manifestation of disease symptoms, most therapies target calpains at the postsymptomatic stage. To identify useful clinical treatments, the efficacy and safety of these therapies should be evaluated using model systems that reflect the symptomatic context of the disease.

As discussed above, achieving specificity when targeting calpains is challenging. Indeed, many of the first-generation calpain inhibitors were nonspecific and targeted other proteases[118]. A calpain inhibitor can be useful for disease treatment, for purely scientific experiments or both. The difference is exemplified by *in vitro* specificity versus practical *in vivo* effects[198]. *In vitro* specificity is gradually being addressed by inhibitor chemistry inspired by structures and other new approaches (see above and BOX 1). Promising strategies include restricting secondary structures of inhibitors[138,139,188,192], learning from the 3D structure of calpain–CAST complexes[188,192], allosteric inhibition, including the idea of intercalation between PC1–PC2 or PEF(L)–PEF(S), and use of cathepsin-specific inhibitors[247,248].

Understanding calpain substrate specificities is another key concern, particularly when considering potential off-target effects when attempting to target calpains. However, this knowledge is lacking, and solving calpain substrate specificity remains a key priority in the field. As discussed above, CysPc domains are structurally nonspecific with respect to recognizing substrate amino acid residue side chains[64,65]. Although bioinformatics studies have achieved practically usable predictors for calpain substrate cleavage sites[98–102], a constitutive principle of how calpains recognize substrates is far from clear.

Another consideration is that *in vivo*, calpain inhibition alone may not be completely effective in treating a disease. Rather, a strategy combining calpain inhibition and other therapeutic protocols may be more beneficial. For this approach, the molecular context of functions of the targeted calpain, including the presence of other drugs and the disease condition, needs to be thoroughly examined. The same principle holds true in designing gene therapies, and information about how the responsible calpains function needs to be constantly updated. Recent reports showing additive effects of calpain inhibition and other drugs are promising examples of this strategy: for example, MDL28170 in combination with the existing ALS therapeutic drug riluzole[249], or genetic disruption plus an HSP90 inhibitor[29].

Conclusions and perspectives

Proteases have proved to be effective targets in various diseases[250]. However, the paucity of information about the substrate specificity of calpains as a protease family has severely limited our understanding of calpain functions. This issue has been appreciably overcome in the past decade or so[64,65,75,95,96,98].

When targeting calpains, inhibition, activation and restoration represent therapeutic options depending on the disease. Promising strategies and inhibitors found to date could be improved through continued basic research. Importantly, the specific and selective manipulation of calpain activities is an increasing research trend. As calpain research proceeds, we need to continuously examine whether newly developed calpain inhibitors are applicable for therapy, and whether gene therapies for calpainopathies are a feasible therapeutic option.

The pathological mechanisms of known calpainopathies have also been extensively studied using state-of-the-art techniques combined with mouse genetics and genome-wide analyses. An exception is the poorly understood role of CAPN10 in the pathology of type 2 diabetes mellitus, an important research topic with substantial implications for modern society[251,252].

The targeting of calpains in causative organisms for infectious diseases is highly challenging because the structures of these calpain species are markedly divergent

from those of conventional calpains. There is therefore an urgent need to systematically advance this field to aid the development of potential novel therapies.

Translational research involving calpains is still at the development stage. To advance, we need to learn more about the calpains themselves, as well as their impact on various physiological systems and molecular pathways and events. The ambiguous impression we have of calpains may simply reflect the fact that they have not yet been thoroughly studied. Calpain molecules actually possess many features that make them attractive subjects for intensive analysis in the field of protein science. A multidisciplinary approach to unveiling the physiological functions of calpains will continue to provide valuable information for medical and basic biological studies. Such knowledge will improve the likelihood that we will successfully correct the aberrant functions of calpains in various disease conditions.

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DATABASES

MEROPS: http://merops.sanger.ac.uk
RCSB Protein Data Bank: http://www.rcsb.org/pdb

FURTHER INFORMATION

Joint Genome Institute: MycoCosm: http://genome.jgi.doe.gov/programs/fungi/index.jsf
Leiden Muscular Dystrophy: Calpain-3 (CAPN3): www.dmd.nl/capn3_home.html

SUPPLEMENTARY INFORMATION

See online article: S1 (figure) | S2 (table)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

Author biographies

Yasuko Ono is Associate Director of the Calpain Project, Tokyo Metropolitan Institute of Medical Science (Igakuken), Japan. Ono received a Ph.D. in protein science from the University of Tokyo, Japan, studying CAPN3 in the laboratory of Koichi Suzuki. In 1999, Ono began postdoctoral training in the field of muscle proteins in the laboratory of Carol C. Gregorio at the University of Arizona, USA, and, in 2004, joined the current research project led by Hiroyuki Sorimachi. Her major research interests include the role of calpain in muscle with an emphasis on physiology and evolution.

Takaomi C. Saido is Senior Team Leader, Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Japan. Saido received a Ph.D. from the Graduate School of Pharmaceutical Science, the University of Tokyo, Japan, in 1988 and then became a research scientist at the Tokyo Metropolitan Institute of Medical Science, Japan, where he mainly worked on the biochemistry and pathophysiology of calpain. Since moving to RIKEN in 1997, he has been working both on calpain and Alzheimer disease. The *Cast* knockout, *Capn2* (conditional) knockout and *App* knock-in mice generated in his laboratory are widely used in the research community.

Hiroyuki Sorimachi started working on calpains in 1988 in Koichi Suzuki's laboratory at the Tokyo Metropolitan Institute of Medical Science (Rinshoken/Igakuken), Japan. In 1992, he received a Ph.D. from the University of Tokyo, Japan, after which he was appointed an assistant professor, and, in 1997, became an associate professor. In 2004, he returned to Rinshoken/Igakuken as Project Leader of the "Calpain Project", and, in 2008, he became a department head. He was awarded a Lifetime Achievement Award from the FASEB SRC Calpain meeting in 2016. His research interests include the biochemistry and genetics of all types of calpains.

Key points

- The calpains are a family of proteases with biologically vital functions. However, the mechanistic features of calpains are largely unknown.
- Calpains have been identified as potential therapeutic targets for various types of diseases, including neurodegenerative and cardiovascular disorders, ophthalmic diseases and cancer.
- Many disease phenotypes are ameliorated by calpain inhibition, and some calpain inhibitors have entered clinical trials.
- Many calpain orthologues in parasites or microorganisms are responsible for the pathogenicity and viability of the organism; thus, targeting these calpains is a promising approach for combatting infectious diseases.
- Some calpain gene defects resulting in loss of calpain activity are pathologically implicated in human disease. Therefore, in addition to therapies that inhibit calpain activity, developing strategies that compensate for calpain loss are an important goal.
- The development of inhibitors with improved efficiency and specificity for calpains is a critical future research direction. Unveiling the physiological functions of calpains at the molecular level is a key challenge.

Subject categories

Biological sciences / Drug discovery
[URI /631/154]
Biological sciences / Chemical biology / Proteases
[URI /631/92/468]
Health sciences / Diseases / Neurological disorders / Neurodegenerative diseases
[URI /692/699/375/365]
Health sciences / Diseases / Cardiovascular diseases
[URI /692/699/75]
Health sciences / Oncology / Cancer
[URI /692/4028/67]
Health sciences / Diseases / Infectious diseases
[URI /692/699/255]

ToC blurb

000 **Calpain research for drug discovery: challenges and potential**

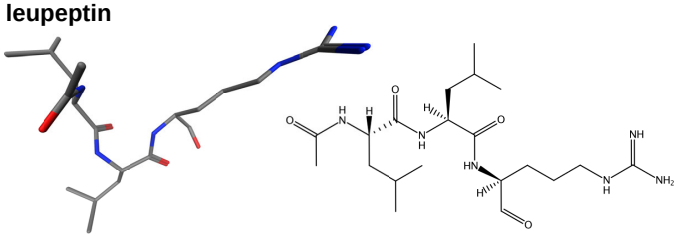
Yasuko Ono, Takaomi C. Saido and Hiroyuki Sorimachi

The calpain family of proteases are involved in numerous physiological and pathological processes. Here, Sorimachi and colleagues provide an overview of the calpain superfamily and calpain-related disorders, assess the various emerging approaches for therapeutically targeting calpains and highlight agents currently in clinical trials.

In format provided by Ono *et al.* (doi:10.1038/nrd.2016.212)

Supplementary Figure 1. Calpain inhibitors and related molecules

1. leupeptin



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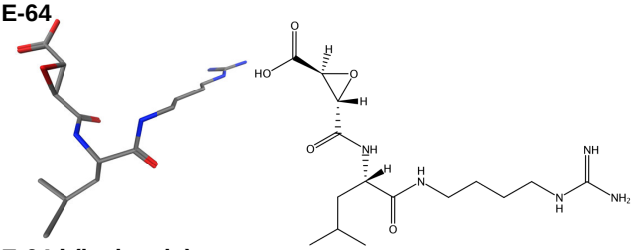

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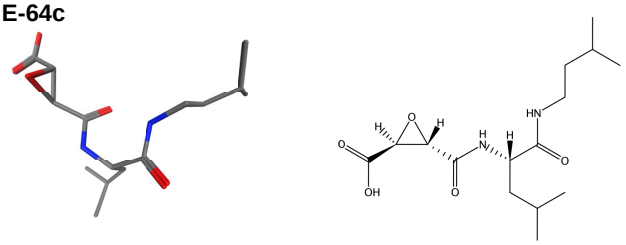

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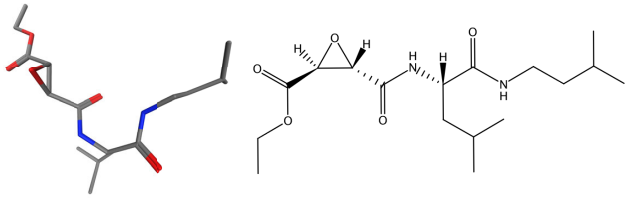

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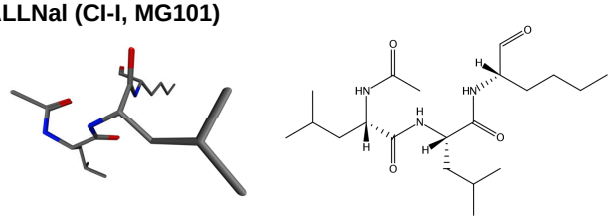

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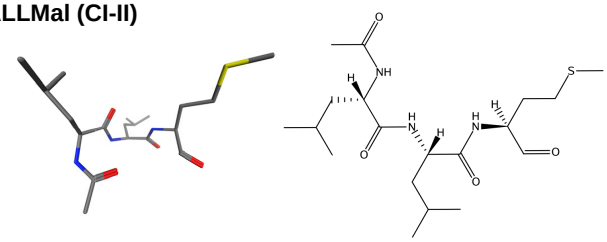

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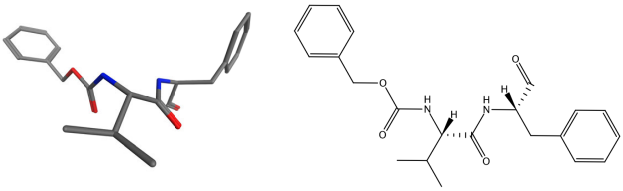
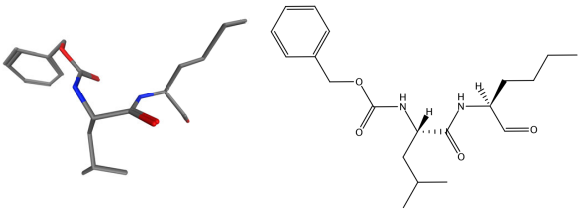


8. calpeptin



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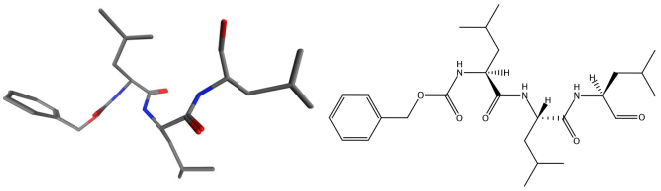

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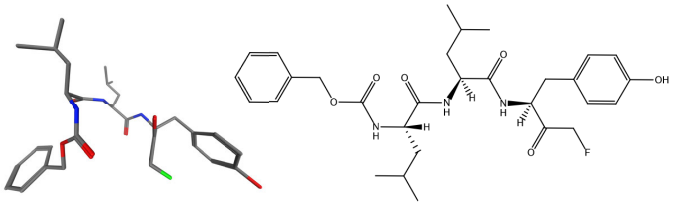

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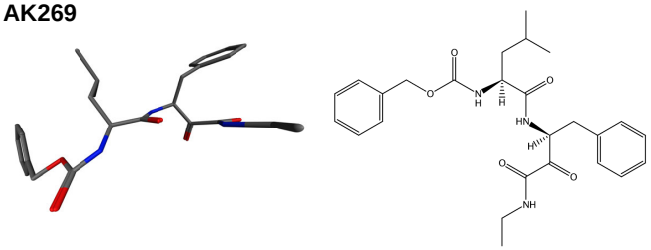

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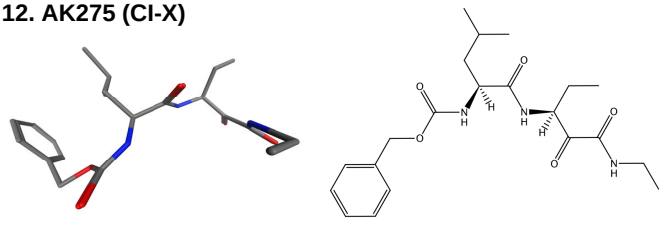

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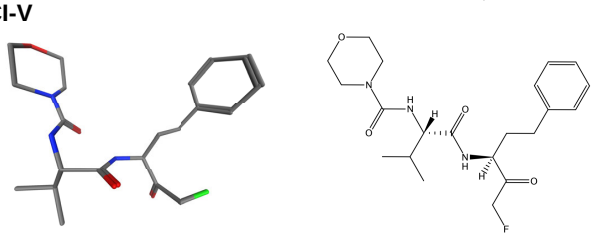

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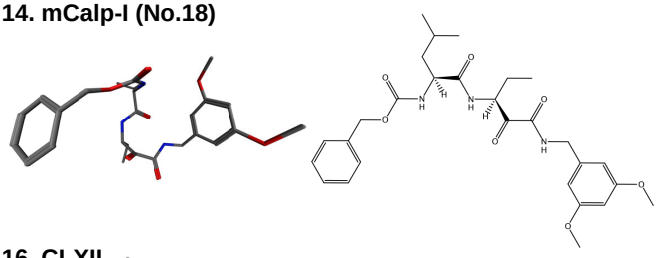

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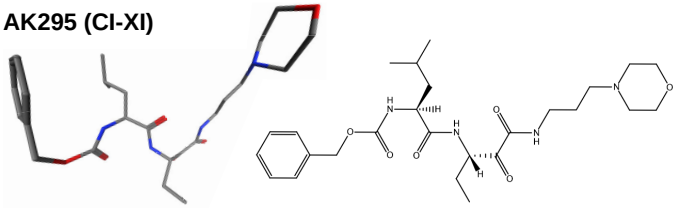

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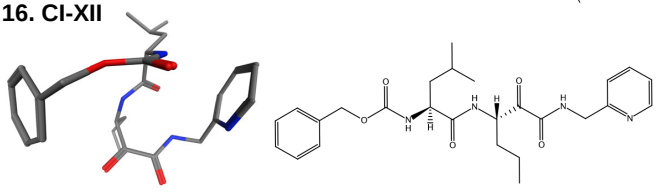

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Supplementary Figure 1 (continued)

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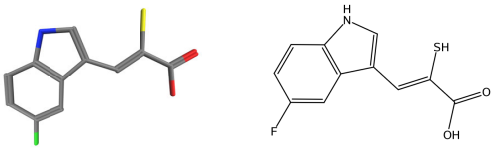
18. PD151746



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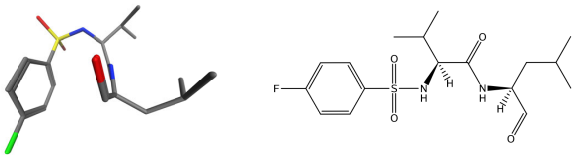

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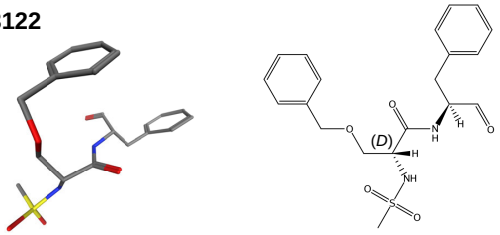

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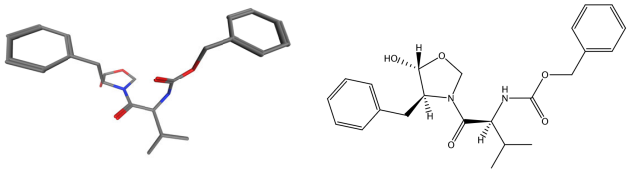

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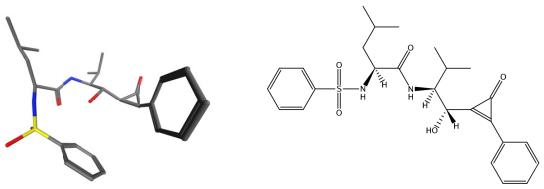

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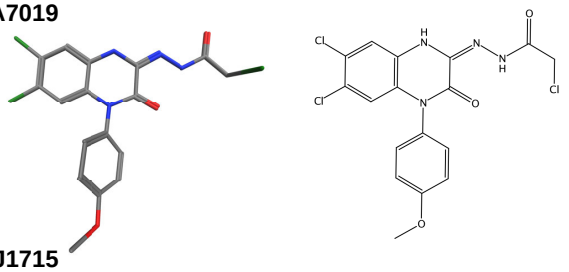

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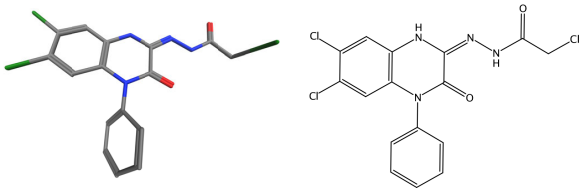

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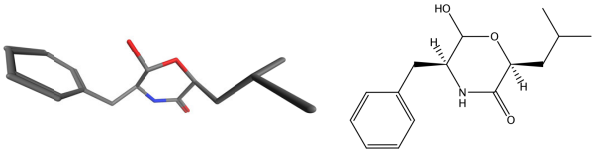
26. SNJ1757



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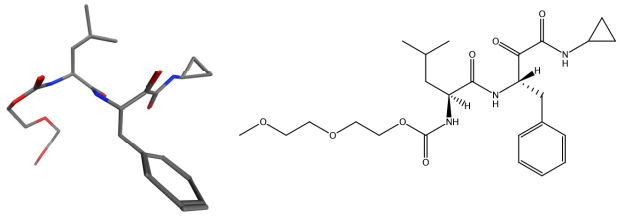

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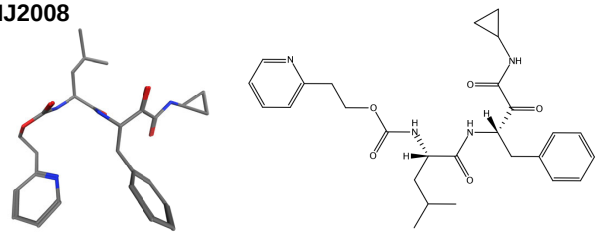

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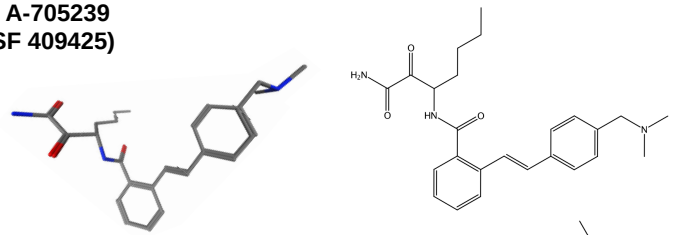

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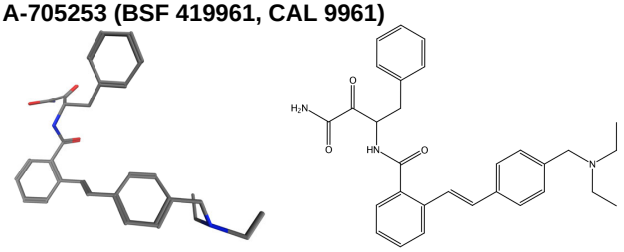

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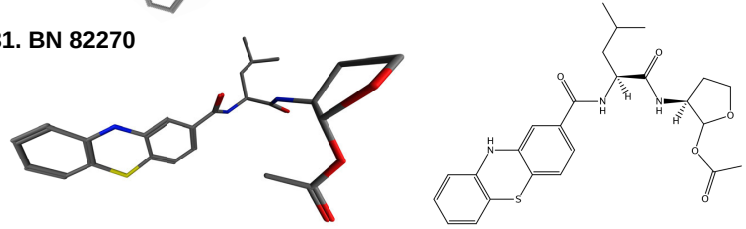

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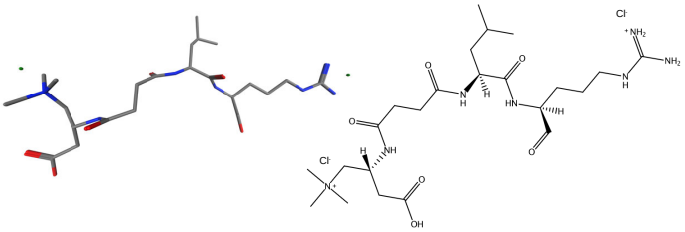
Supplementary Figure 1 (continued)

32. C-101 (Myodur)



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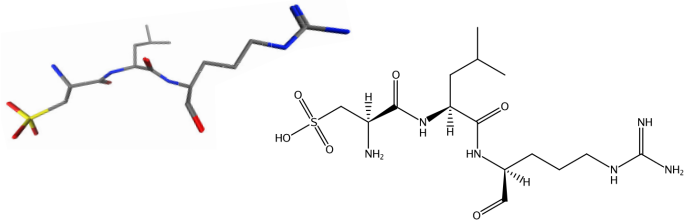

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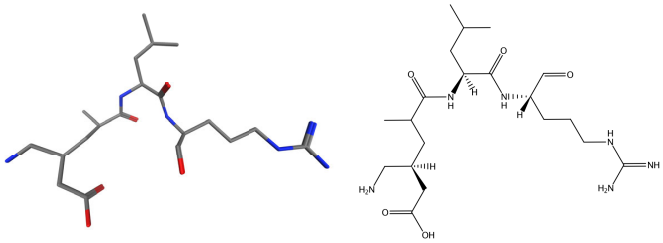

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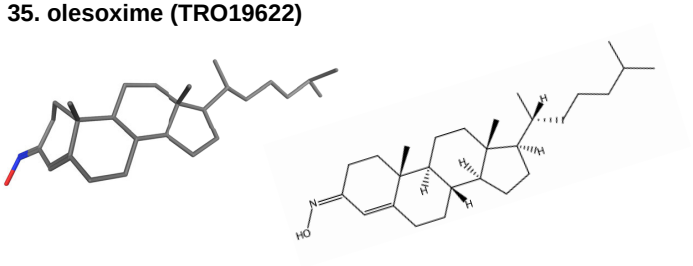

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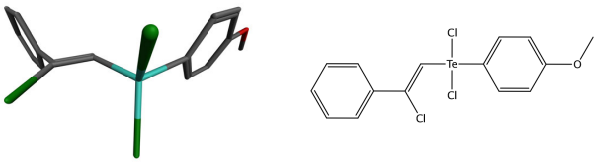
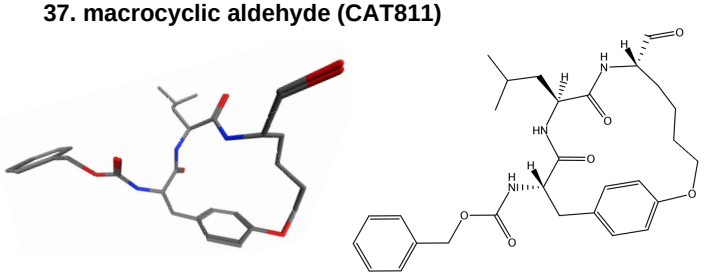
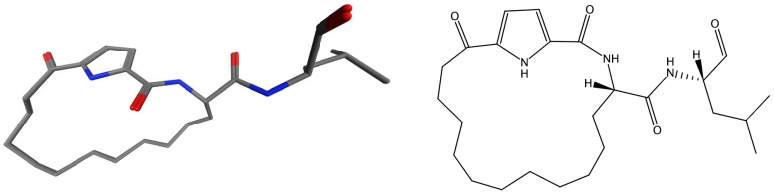


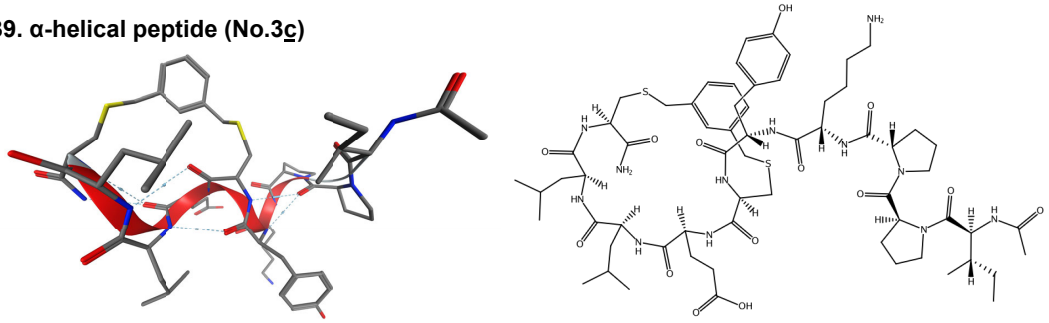
37. macrocyclic aldehyde (CAT811)



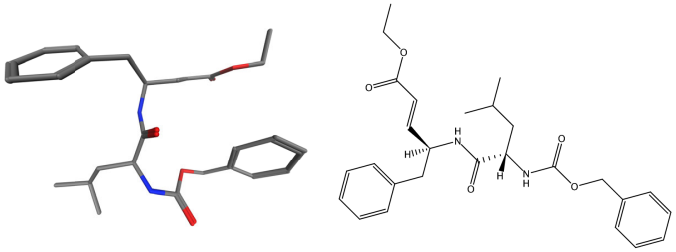
38. indole-containing 18-membered aldehyde (No. 1c)



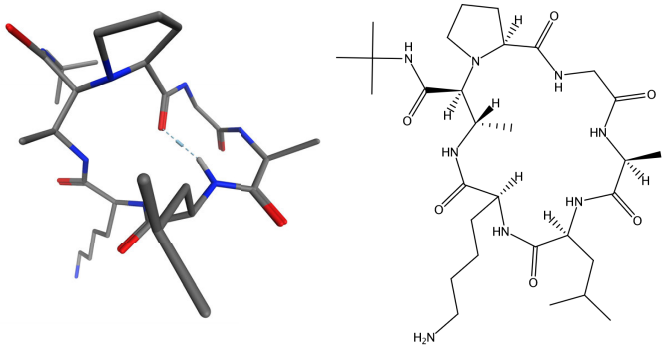
39. α-helical peptide (No.3c)



40. dipeptidyl α,β-unsaturated ester (No.8)



41. macrocyclic β-turn peptide (c*[PGALK])



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Supplementary figure legend

Supplementary Figure 1: Calpain inhibitors and related molecules

Examples of calpain inhibitors and related molecules are numbered here approximately in the order of their discovery/synthesis (the earliest discovered are at the top, as in Supplementary Table 1). Note that they are not actually specific for calpains. Descriptive names of the smaller molecules are as follows (for their properties, see Supplementary Table 1):

1. leupeptin, Ac-*L*-Leu-*L*-Leu-*L*-argininal[1]

2. E-64, [(2*S*,3*S*)-3-carboxyoxirane-2-carbonyl]-*L*-Leu-(4-guanidinobutyl)amide)[2,3]

3. E-64c, [(2*S*,3*S*)-3-carboxyoxirane-2-carbonyl]-*L*-Leu-(3-methylbutyl)amide[4]

4. E-64d (loxistatin), [(2*S*,3*S*)-3-ethoxycarbonyloxirane-2-carbonyl]-*L*-Leu-(3-methylbutyl)amide)[4]

5. ALLNal (calpain inhibitor (CI-) I, MG101), Ac-*L*-Leu-*L*-Leu-*L*-norleucinal[5]

6. ALLMal (CI-II), Ac-*L*-Leu-*L*-Leu-*L*-methional[5]

7. MDL28170 (CI-III), Z-*L*-Val-*L*-phenylalaninal[6]

8. calpeptin, Z-*L*-Leu-*L*-norleucinal[7]

9. MG132, Z-*L*-Leu-*L*-Leu-*L*-leucinal[8]

10. CI-IV, Z-*L*-Leu-*L*-Leu-*L*-Tyr-CH_2F[9,10]

11. AK269, Z-*L*-Leu-*L*-Phe-CONH-C_2H_5[11-13]

12. AK275 (CI-X), Z-*L*-Leu-*L*-Abu-CONH-C_2H_5[11-15]

13. CI-V, morpholinoureidyl-*L*-Val-*L*-homophenylalanyl-CH_2F[16]

14. mCalp-I (No. 18), Z-*L*-Leu-*L*-Abu-CONH-CH_2-C_6H_3-3,5-$(OCH_3)_2$[13]

15. AK295 (CI-XI), Z-*L*-Leu-*L*-Abu-CONH-$(CH_2)_3$-morpholine[12,13,17]

16. CI-XII, Z-*L*-Leu-*L*-norvaline-CONH-CH_2-2-pyridyl[18]

17. PD150606, 3-(4-iodophenyl)-2-mercapto-(*Z*)-2-propenoic acid[19]

18. PD151746, 3-(5-fluoro-3-indolyl)-2-mercapto-(*Z*)-2-propenoic acid[19]

19. SJA6017 (CI-VI), 4-fluorophenylsulfonyl-*L*-Val-*L*-leucinal[20]

20. CEP-3122, CH_3-SO_2-*D*-phenylmethylserine-*L*-phenylalaninal[21]
(**CEP-3453** is the HSO_3 addition of CEP-3122[22])

21. MDL104903, [1-[(5-hydroxy-4-phenyl-methyl-3-oxazolidinyl)carbonyl]-2-ethylpropyl]carbamic acid phenylmethyl ester[23]

22. BDA-410, (2*S*)-N-[(1*S*)-1-[(*S*)-hydroxy(3-oxo-2-phenyl-1-cyclopropen-1-yl)methyl]-2-methyl propyl]-2-benzenesulfonylamino-4-methylpentanamide[24-26]

23. SJA7019, chloroacetic acid *N*'-[6,7-dichloro-4-(4-methoxyphenyl)-3-oxo-3,4-dihydro quinoxalin-2-yl]hydrazide[27]

24. SJA7029, chloroacetic acid *N*'-(6,7-dichloro-4-phenyl-3-oxo-3,4-dihydroquinoxalin-2-yl) hydrazide[27]

25. SNJ1715, (2*S*)-4-methyl-2-(3-phenylthioureido)-N-[(3*S*)-tetrahydro-2-hydroxy-3-furanyl] pentanamide[28]

26. SNJ1757, (2*S*,5*S*)-5-benzyl-6-hydroxy-2-(2-methylpropyl)-3-morpholinone[29]

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27. SNJ1945, [(1*S*)-1-([((1*S*)-1-benzyl-3-(cyclopropylamino)-2,3-dioxopropyl)amino]carbonyl)-3-methylbutyl]carbamic acid 5-methoxy-3-oxapentyl ester[30]

28. SNJ2008, [(1*S*)-1-[([(1*S*)-1-benzyl-3-(cyclopropylamino)-2,3-dioxopropyl]amino)carbonyl]-3-methylbutyl]carbamic acid 2-(pyridin-2-yl)ethyl ester[31]

29. A-705239 (BSF 409425), *N*-(1-carbamoyl-1-oxohex-1-yl)-2-[*E*-2-(4-dimethylaminomethyl phenyl)ethen-1-yl]benzamide[32]

30. A-705253 (BSF 419961, CAL 9961), *N*-(1-benzyl-2-carbamoyl-2-oxoethyl)-2-[*E*-2-(4-diethyl aminomethylphenyl)ethen-1-yl]benzamide[32]

31. BN 82270 (No. 7), phenothiazine-*L*-Leu-2-hydroxytetrahydrofuran[33]

32. C-101 (Myodur), *L*-aminocarnitylsuccinyl-*L*-Leu-*L*-argininal dichloride[34] (Counter ions (Cl^-) are depicted as associated, but not covalently connected, objects in the structures).

33. C-201 (Neurodur), *L*-cysteyl-*L*-Leu-*L*-argininal[35] (**CYLA** is diethyl acetal of C-201[36])

34. GABAdur, pregabalin-*L*-Leu-*L*-argininal[37]

35. olesoxime (TRO19622), (3*Z*)-*N*-hydroxycholest-4-en-3-imine[38]

37. macrocyclic aldehyde (No. 2d, CAT811), ((7*S*,10*S*,13*S*)-7-formyl-10-isobutyl-9,12-dioxo-2-oxa-8,11-diaza-bicyclo[13.2.2]nonadeca-1(18),15(19),16-trien-13-yl)-carbamic acid benzyl ester[39]

38. indole-containing 18-membered aldehyde (No. 1c), (*S*)-*N*-[(*S*)-4-methyl-1-oxopentan-2-yl]-2,16-dioxo-3,20-diazabicyclo[15.2.1]icosa-l(19),17-diene-4-carboxamide[40]

39. dipeptidyl α,β-unsaturated ester (No. 8), (*E*)-ethyl-4-(2-(benzyl-oxycarbonyl-amino)-4-methyl pentanamido)-5-phenylpent-2-enoate[41].

For the following larger molecules, molecular ID used in the first paper reporting their synthesis is as follows:

36. hypervalent organotellurium compound, No. 11[42] or RF19[43]

39. α-helical peptide (Ac-IPPKYCELLC-NH_2), No. 3c[44]

41. macrocyclic β-turn peptide (PGALK), c*[PGALK][45].

Stick representations (C: grey, N: blue, O: rouge, F: lime, S: yellow, Cl: green, I: dark red, Te: turquoise, H: not shown) and 2D chemical structures were energy minimized and drawn by MOE Ver. 2015.10. In the structure of 39 and 41, a ribbon scheme and/or hydrogen bonds are shown.

Abbreviations: Abu, α-aminobutylic acid residue; Ac, acetyl; Z, benzyloxycarbonyl.

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Supplementary table

Supplementary Table 1: Properties of calpain inhibitors

Ki and IC50 values vary substantially depending on the reference. The values shown here are from the references indicated. Numbers (No.) correspond to those in Supplementary Figure 1.

No.	Inhibitor	Composition Formula	Mr	Year[*1]	Mode[*2]	Inhibitory activity (upper: Ki, lower: IC50 if otherwise indicated) (nM)							Ref.
						C1[*3]	C2[*3]	papain	CathB[*4]	CathL	Trypsin	Others	
1	Leupeptin	$C_{20}H_{38}N_6O_4$	426.56	1969	R	320 351	430 176	- $1.5x10^3$	6 27		35 -	plasmin: $Ki=3.4x10^3$ kalikrein: $Ki>1.9x10^5$	1,5,20, 29,46,47
2	E-64	$C_{15}H_{27}N_5O_5$	357.41	1978	I	800[*5] $1.0x10^3$	- $2.6x10^3$	- 290	- 800				2,48
3	E-64c	$C_{15}H_{26}N_2O_5$	314.38	1980	I	960[*5] $1.5x10^4$ [*5]		- $4.4x10^3$	- 240	- 90			3,4,48
4	E-64d (loxistatin)	$C_{17}H_{30}N_2O_5$	342.44	1986	I							(active only after hydrolysis (=E-64c))	4
5	ALLNal (CI-I, MG101)	$C_{20}H_{37}N_3O_4$	383.53	1986	R	86 310	192 160	$2.2x10^3$ $4.5x10^3$	22 -		$>5x10^5$ -	thermolysin: $Ki>5x10^5$ MMP2: $IC50=2.19x10^4$ calcineurin/CaM: $Ki>2x10^5$	5,19,49
6	ALLMal (CI-II)	$C_{19}H_{35}N_3O_4S$	401.57	1986	R	- 250	- 140	- $3.6x10^3$					5
7	MDL28170 (CI-III)	$C_{22}H_{26}N_2O_4$	382.46	1988	R	7 -	- 100		25 -		$>2x10^6$ -	plasmin: $Ki=2.7x10^5$ kalikrein: $Ki>5x10^6$	6,46,50
8	Calpeptin	$C_{20}H_{30}N_2O_4$	362.47	1988	R	- 52	- 34	- 138				Proteasome: $Ki=1.1x10^5$ Immunoproteasome: $Ki=1.0x10^3$	7,51
9	MG132	$C_{26}H_{41}N_3O_5$	475.63	1991	R		10 $1.2x10^3$					20S proteasome: IC50=100, $Ki=6.9x10^3$	8,51,52
10	CI-IV	$C_{30}H_{40}N_3O_6F$	557.66	1991	I		k[*6]= $2.9x10^4$ [*5]			k= $6.8x10^5$			9,10
11	AK269	$C_{26}H_{33}N_3O_5$	467.57	1993	R	200 -	39 -	$4.5x10^4$ -	$6.0x10^3$ -				11,18
12	AK275 (CI-X)	$C_{21}H_{31}N_3O_5$	405.50	1993	R	250 -	210 -	$9.3x10^4$ -	$2.4x10^3$ -				11,18
13	CI-V	$C_{21}H_{30}N_3O_4F$	407.49	1994	I				- ~10	- ~100		*ca.*70% inhibition of total cysteine protease activity from rat kidney by oral administration (10.0mg/kg).	16,53
14	mCalp-I (No. 18)	$C_{28}H_{37}N_3O_7$	527.62	1996	R	940 $2.3x10^3$	25 22		- $1.8x10^3$				18,54
15	AK295 (CI-XI)	$C_{26}H_{40}N_4O_6$	504.63	1996	R	140 -	41 -		$6.9x10^3$ -				18
16	CI-XII	$C_{26}H_{34}N_4O_5$	482.58	1996	R	19 -	120 -		750 -				18
17	PD150606	$C_9H_7O_2SI$	306.12	1996	R	210 -	370 -	$>5x10^5$ -	$1.3x10^5$ -		$>5x10^5$ -	thermolysin: $Ki=2.0x10^5$ MMP2: $IC50=9.3x10^3$ calcineurin/CaM: $Ki=1.3x10^4$	19,49
18	PD151746	$C_{11}H_8NO_2SF$	237.25	1996	R	260 -	$5.3x10^3$ -	$>5x10^5$ -	$>2x10^5$ -		$>5x10^5$ -	thermolysin:$Ki>5x10^5$ calcineurin/CaM: $Ki>8.5x10^4$	19
19	SJA6017 (CI-VI)	$C_{17}H_{25}N_2O_4SF$	372.46	1997	R	- 22	- 49		- 6.9			20S proteasome: $IC50>10^5$	20,28,29
20	CEP-3122	$C_{20}H_{24}N_2O_5S$	404.49	1998	R	8 -	5 -	32 -				IC50 of CEP3453 for C1: Ki=15, for CathB: Ki=15	21,22
21	MDL104903	$C_{23}H_{28}N_2O_5$	412.49	1999	R	33 -							23
22	BDA-410	$C_{26}H_{32}N_2O_5S$	484.62	1999	R	130 21	630 21	- 400	- $1.6x10^5$			thrombin, CathG, proteasome: $Ki=1.0x10^5$ CathD: $Ki=9.1x10^5$	24,25,55
23	SJA7019	$C_{17}H_{13}N_4O_3Cl_3$	427.67	1999	I	- 77	- 64			- $1.5x10^3$			27
24	SJA7029	$C_{16}H_{11}N_4O_2Cl_3$	397.65	1999	I	- 120	- 170			- $4.2x10^3$			27
25	SNJ1715	$C_{17}H_{25}N_3O_3S$	351.47	2003	R	- 86	- 190					20S proteasome: $IC50>10^5$	28
26	SNJ1757	$C_{15}H_{21}NO_3$	263.34	2003	R	- 700	- 930		- $>1x10^5$				29

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27	SNJ1945	$C_{25}H_{37}N_3O_7$	491.58	2005	R	- 170	- 99						30
28	SNJ2008	$C_{27}H_{34}N_4O_5$	494.59	2006	R	- 29	- 17						31,56
29	A-705239 (BSF 409425)	$C_{25}H_{31}N_3O_3$	421.54	2003	R	13 -			27 -	22 -		proteasome: Ki=4.0x10^5	32,57
30	A-705253 (BSF 419961, CAL 9961)	$C_{30}H_{33}N_3O_3$	483.61	2003	R	27 -			62 -	149 -		proteasome: Ki=2.6x10^4	32,57
31	BN 82270	$C_{25}H_{29}N_3O_5S$	483.59	2004	R	- >1x10^3						Dual inhibitor for calpains (active after hydrolysis of the acetyl group) and lipid peroxidation. Cellular calpain inhibition: IC50=1.33x10^4; Fe^{2+} induced lipid peroxidation in rat brain microsomes inhibition: IC50=1.55x10^4; permanent hearing and hair cell loss of guinea pigs induced by sound trauma: IC50=4x10^3	33,58
32	C-101 (Myodur)	$C_{23}H_{47}N_7O_6Cl_2$	588.58	2005	R							spectrin breakdown (145 kDa): IC50=3.7x10^4	34
33	C-201 (Neurodur)	$C_{15}H_{30}N_6O_6S$	422.51	2005	R							(no Ki or IC50 data)	35
-	CYLA	$C_{19}H_{40}N_6O_7S$	496.63	2006	R							Total calpain amounts in spinal cord homogenates were reduced *ca.* a half after 2 mg/mouse/day administration. (active only after hydrolysis (=C-201))	36
34	GABAdur	$C_{20}H_{38}N_6O_5$	442.56	2007	R							(no Ki or IC50 data)	37
35	Olesoxime (TRO19622)	$C_{27}H_{45}NO$	399.66	2007	R	*7	*7					mitochondrial translocator protein 18 kDa and its ligand, PK11195, binding: IC50=3~5x10^4	38,59,60
36	Hypervalent organotellurium compound (No. 11, RF19)	$C_{15}H_{13}OCl_3Te$	443.23	2009	I	- 200*8			*k*= 7.9 x10^3	*k*= 9.4x10^3		CathS: *k*=2.0 x10^5 CathK: *k*=3.8 x10^5	42,43
37	Macrocyclic aldehyde (CAT811)	$C_{29}H_{37}N_3O_6$	523.63	2009	R	- 220	- 30	- >5x10^5	- 70			pepsin and α-chymotrypsin: IC50>5x10^5	39
38	Indole-containing 18-membered aldehyde (No.1c)	$C_{25}H_{39}N_3O_4$	445.60	2012	R	- 42	- 66					α-chymotrypsin: IC50>2.5x10^5	40
39	α-helical peptide (No. 3c, Ac-IPPKYCELLC-NH_2)	$C_{64}H_{96}N_{12}O_{14}S_2$	1321.67	2012	R	1x10^4 -		>1x10^5 -	>1x10^5 -	3.9x10^4 -			44
40	Dipeptidyl α,β-unsaturated ester (No.8)	$C_{27}H_{34}N_2O_5$	466.58	2012	R							IC50 for *P. falciparum* growth rate in human erythrocytes=5x10^3 IC50 for HeLa cell growth=3.5x10^5	41
41	Macrocyclic β-turn peptide (c*[PGALK])	$C_{30}H_{54}N_8O_6$	622.81	2016	R	(~50%*9)	1.7x10^4 -	(~10%)		(~100%)			45

*1: a year of the first report for synthesis or discovery; *2: a mode of inhibition, reversible (R) or irreversible (I); *3: C1, calpain-1; C2, calpain-2; *4: Cath, cathepsin; *5: for chicken calpain-11; *6: a rate constant ($M^{-1}s^{-1}$) of a reaction, E+I→EI (E: enzyme, I: inhibitor); *7: showing *in vivo* inhibitory activity; *8: for *P. falciparum* proteases; *9: relative to inhibitory effect observed for C2 under the same condition.

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Notes for references

[1]In the original report, leupeptin was shown to have *DL*-argininal; however, a later study[61] showed that *L*-argininal, but not *D*-argininal, has a strong affinity to trypsin. Thus, the structure is shown as Ac-*L*-Leu-*L*-Leu-*L*-argininal.

[5]The original report was by Saito, M., Higuchi, N., Kawaguchi, N., Tanaka, T. and Murachi, T. at the 4th FAOB Congress in Singapore, November 30, 1986 (Abstracts of Papers, p58).

[11,18]The AK275 was first synthesized as a diastereometric mixture (called CX275); however, since a later study[14] showed that the *L*, *L* isomer has inhibitory activity, this active structure is shown here, and so are for other AK series inhibitors.

[16]This reference described synthesis and the effect of morpholinoureidyl-*L*-**Leu**-*L*-homophenylalanyl-CH_2F (mu-**L**-hF-fmk, P34089), but not those of **Val** (mu-**V**-hF-fmk, *i.e.*, CI-V). Since the described method can be applied to mu-**V**-hF-fmk (CI-V) and no other reference describing the synthesis of CI-V was found, this reference is cited here.

[27]The original report was by Inoue, J., Cui, Y.-S., Sakai, O., Nakamura, M., Yuen, P.-W., and Wang, K.K.W. (1999) α-Substituted hydrazides having calpain inhibitory activity, in *Proceedings of the FASEB Summer Conference on Calpains*, 1999 June 20–23, Breckenridge, CO.

[34]This reference did not mention about the stereochemical analysis, and, thus, the structure is an estimate from those of *L*-carnitine and leupeptin.

[35]This reference did not mention about the stereochemical analysis, and, thus, the structure is an estimate from those of *L*-cysteic acid and leupeptin.

[37]This reference did not mention about the stereochemical analysis, and, thus, the structure is an estimate from those of pregabalin and leupeptin.