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REVIEW

Calpain as a therapeutic target in cancer

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ABSTRACT

Introduction: Calpain-1 and calpain-2 are prototypical classical isoforms of the calpain family of calcium-activated cysteine proteases. Their substrate proteins participate in a wide range of cellular processes, including transcription, survival, proliferation, apoptosis, migration, and invasion. Dysregulated calpain activity has been implicated in tumorigenesis, suggesting that calpains may be promising therapeutic targets.

Areas covered: This review covers clinical and basic research studies implicating calpain-1 and calpain-2 expression and activity in tumorigenesis and metastasis. We highlight isoform specific functions and provide an overview of substrates and cancer-related signalling pathways affected by calpain-mediated proteolytic cleavage. We also discuss efforts to develop clinically relevant calpain specific inhibitors and spotlight the challenges facing inhibitor development.

Expert opinion: Rationale for targeting calpain-1 and calpain-2 in cancer is supported by pre-clinical and clinical studies demonstrating that calpain inhibition has the potential to attenuate carcinogenesis and block metastasis of aggressive tumors. The wide range of substrates and cleavage products, paired with inconsistencies in model systems, underscores the need for more complete understanding of physiological substrates and how calpain cleavage alters their functions in cellular processes. The development of isoform specific calpain inhibitors remains an important goal with therapeutic potential in cancer and other diseases.

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Apoptosis; calpain; calpastatin; cancer; CAPN; invasion; metastasis; migration; protease; survival

1. Introduction

First identified in the 1960s, calpain-1 and calpain-2 are the founding members of a family of calcium (Ca^{2+})-dependent cysteine proteases that are being explored as possible therapeutic targets in diseases, including Alzheimer's and several types of cancer [1,2]. A growing body of literature, spanning several cancer subtypes, supports roles for calpains in tumorigenesis and disease progression. With a diverse range of known calpain substrates involved in different cellular and physiological functions, the precise roles of calpains in different cancers appear complex and, in some cases, paradoxical. This review aims to discuss the rationale for calpain inhibition as a therapeutic strategy in cancer. We begin by summarizing the structure and regulation of calpain. We then examine translational studies focused on calpain dysregulation, biologically relevant calpain substrates, and the cellular processes they are involved in. We also discuss ongoing efforts to develop pharmacologic calpain inhibitors and the limitations that must be addressed to realize the full potential of these as therapeutic agents. We regret that we are not able to describe or acknowledge every publication that has contributed to our current understanding of the calpain system in cancer biology. For other recent reviews on the subject, we refer the reader to the following publications [3-7].

1.1. Structure and activation of conventional calpains

Human calpains are a family of 15 Ca^{2+}-activated cysteine proteases which seemingly cleave disordered or accessible peptide sequences, rather than targeting specific amino acid motifs, and thus have a myriad of peptide substrates [8] . The precise biologic roles of calpains are elusive, with evidence pointing towards more than 130 substrate proteins [9] involved in a wide range of cellular functions; many of which are discussed in this review.

The conventional calpain isoforms, calpain-1 and −2 (previously known as μ-calpain and m-calpain) were the first to be discovered and are the most well studied due to their abundant ubiquitous expression. Both isoforms are intracellular heterodimers consisting of a common regulatory subunit, encoded by the *CAPNS1* gene (also known as *CAPN4*), and an isoform-specific catalytic subunit encoded by the *CAPN1* or *CAPN2* genes, for calpain-1 and calpain-2, respectively [10,11]. Calpain-1 and −2 are also considered classical calpains due to their defining domain structures. The catalytic subunit consists of an N-terminal anchor helix, a potential regulator of calpain activation [12]; two protease core domains that constitute the active site (PC1 and PC2); the calpain-type beta-sandwich (CBSW) domain (previously known as a C2-like domain); and a Ca^{2+} binding C-terminal penta EF-hand PEF(L) domain, a mediator of dimerization and a distinguishing feature of the classical calpain isoforms. The regulatory subunit CAPNS1 consists of an

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Article Highlights

- Calpains are intracellular calcium-activated cysteine proteases that regulate processes, including cell survival, migration, and invasion, through a wide range of substrates.
- Clinical data show that elevated expression of calpain-1 and calpain-2 isoforms in cancer is associated with shorter survival.
- Genetic abrogation of calpain-1 and calpain-2 in cell and animal models correlates with anti-tumor effects.
- Pharmacological inhibitors of calpain exist, but they often lack specificity. They are not approved for clinical use.
- Many studies indicate that calpain inhibition could protect against tissue and organ damage associated with excessive or chronic inflammation.
- Calpain inhibitor BLD-2660 is undergoing a clinical trial for treatment of COVID-19 to reduce tissue IL-6 and prevent lung fibrosis. Another phase-2 trial of this drug in idiopathic pulmonary fibrosis was recently withdrawn.
- Calpain inhibition has potential side effects. For instance, inhibition of calpain activity could impact processes such as wound repair. Hence, surgery, may need to be planned carefully around calpain therapy.

This box summarizes key points contained in the article.

unstructured glycine rich (GR) domain; and a PEF(S) domain, which is homologous to the PEF(L) domain. A crystal structure of calpain-2 and domain maps for the catalytic (CAPN1/2) and regulatory (CAPNS1) subunits are shown in Figure 1.

Calpain-1 and −2 activities are tightly regulated, with only transient activation of proteolysis upon binding of Ca^{2+} ions [14]. The required concentration of Ca^{2+} for *in vitro* activation is in the low micromolar range for calpain-1, and high micromolar to low millimolar range for calpain-2 [14]. Since the intracellular Ca^{2+} concentration (100 nM) [15] is insufficient for calpain activation, these enzymes must be activated by Ca^{2+} influx from the extracellular space, where Ca^{2+} concentration is 1.1–1.4 mM [16]. Alternatively, calpains-1 and −2 may be activated by Ca^{2+} release from intracellular stores, such as the endoplasmic reticulum (ER) [16]. Due to the cells' propensity to rapidly 'clean up' free cytoplasmic Ca^{2+}, calpains-1 and −2 are believed to be transiently activated by localized bursts of adequate concentrations of Ca^{2+}, followed quickly by deactivation after Ca^{2+} levels dissipate. Excessive calpain activation is believed to occur under disease or tissue damage conditions as a result of dysregulated Ca^{2+} homeostasis [8].

Calpain-1 and −2 are also regulated by calpastatin (encoded by the *CAST* gene) which binds the PEF(L) and PEF(S) domains as well as near the active site to sterically hinder substrate access (Figure 2) [17]. The calpain-calpastatin system is regulated by phosphorylation modifications of either calpain or calpastatin. Phospho-CAST has repressed activity, and as such, dephosphorylation is required for CAST to inhibit calpain [18,19]. Phosphorylation of calpain may result in activation or inhibition depending on the kinase. For example, ERK- and protein kinase C-mediated phosphorylation of calpain-2 increases its activity, while phosphorylation by protein kinase A (PKA) decreases calpain-2 activity [20,21]. Our understanding of calpain-calpastatin cross-talk in normal health and diseases, including cancer, is incomplete. There is evidence in glioblastoma cell lines that radiation-induced CAST phosphorylation is associated with activation of calpain-1 and increased cell survival [22]; and while calpain-1 is inhibited by CAST regardless of the calpain-1 phosphorylation status, PKA phosphorylated calpain-1 is more sensitive to CAST inhibition [23]. In addition, the threshold for calpain-2 activation by Ca^{2+} can be reduced by autolysis of the

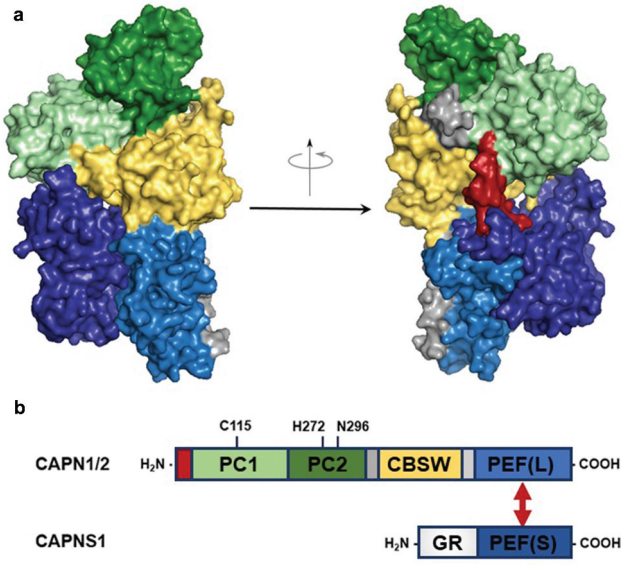

Figure 1. **Structure of calpain-2 and domain diagram for CAPN1/2 and CAPNS1. (A)** A three-dimensional structure of calpain-2 with color-coded domains, created with a structure from PDB 1KFU [13]. Grey molecular surfaces are inter-domain linkers flanking the CBSW domain in CAPN2, and the red surface is the N-terminal anchor helix of CAPN2. The GR domain of CAPNS1 is unstructured and thus not shown. **(B)** A domain diagram of calpain-1/2 showing the red N-terminal anchor helix, PC1 – protease core 1, PC2 – protease core 2, CBSW – calpain-type beta-sandwich, and PEF(L) – penta EF-hand in the catalytic large subunits, CAPN1/2; and the GR – glycine-rich, and PEF(S) – penta EF-hand in the regulatory small subunit, CAPNS1. Amino acids of the catalytic triad are shown with CAPN1 residue numbering. The double-ended red arrow indicates interactions of PEF(L) and PEF(S) mediating dimerization of CAPN1/2 with CAPNS1.

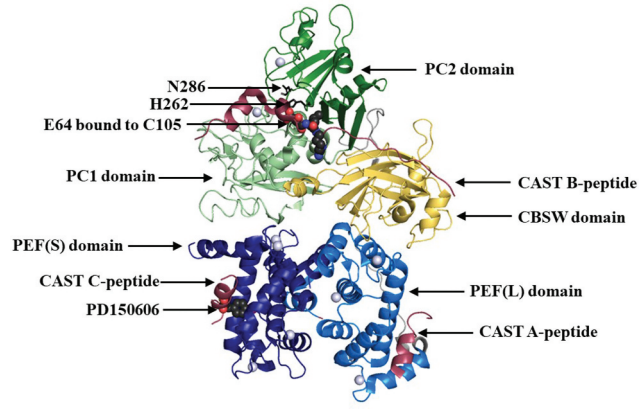

Figure 2. **Binding sites for Ca^{2+}, CAST and small molecule inhibitors of calpain-2**. White spheres represent Ca^{2+} ions. The different domains in CAPN2 and CAPNS1 are colored as in Figure 1. Individual peptide A, B and C motifs of calpastatin (CAST) are shown in crimson, binding to the CAPN2 PEF(L) domain, across the active site, and the CAPNS1 PEF(S) domain, respectively. Molecular structures of inhibitors are shown at the binding positions for two proposed modes of small-molecule inhibition of calpain; active site directed (E64) and allosteric site through binding to the CAPNS1 PEF(S) domain (PD150606). Highlighted residues C105, H262 and N286 comprise the catalytic triad of calpain-2. This 3D representation is based on PDB structures 1KFX [13], 3DF0 [24], 1TLO [25], and 1NX3 [26].

N-terminus or interaction with phosphatidylinositol mono- or bis-phosphate (reviewed in [2]). Binding sites on calpain-2 for Ca^{2+}, calpastatin, and selected small molecule inhibitors (as determined in co-crystal structures) are shown in Figure 2.

Calpain expression levels in cell lysates or *in situ* can be readily assessed by immunoblotting, immunohistochemical, or immunofluorescence methods. However, it is much more challenging to determine the activation status of calpain in cells or tissues. Biochemical zymography methods are used extensively to measure calpain activity in cell lysates [27], but these are semi-quantitative and do not necessarily reflect calpain activity *in situ*. Fluorescent substrates have been developed as tools for measuring calpain activity *in vitro*. These probes typically consist of a fluorophore-quencher pair linked by a calpain-sensitive peptide [28]. Such probes are not completely specific for calpain, so investigators should exercise caution when using cell permeable forms of these probes to assess cellular calpain activity. Fluorescence resonance energy transfer (FRET) probes, consisting of optimal calpain-sensitive peptides bridging CFP and YFP, have also been expressed in cells to measure *in situ* calpain activity [29]. While these can be excellent calpain substrates in cells, there is still some concern that the apparent calpain activity could be due in part to other proteases. Many researchers assess calpain activity in cells by immunoblotting for known calpain substrates and comparing the relative level of the uncleaved substrate with the presumed calpain-generated fragments. While this approach is widely used, it is challenged by the typically low stoichiometry of substrate cleavage, as well as uncertainty about protease specificity, since calpain cleavage sites may also be targeted by other proteases. Measuring physiologically relevant calpain activity *in situ* remains an important challenge in calpain research. The terminal amine isotope labeling of substrates (TAILS) mass spectrometry (MS) approach [30], combined with genetic model systems, has the potential to address these problems and to identify novel physiologically relevant calpain substrates.

2. Calpains

2.1. The role of calpains in human health and disease

The biological function of calpains in human health is multifaceted, which likely reflects diverse roles in cell signaling by 15 isoforms, many of which display distinct tissue-specific expression (reviewed in [8]). Among the first known cellular functions was cleavage of cortical cytoskeletal proteins. Calpain-1 and −2 are involved in turnover of cell adhesion/protrusion structures via proteolytic degradation of scaffolding proteins, such as spectrin, cortactin, ezrin, and beta-catenin (see Table 1 for a list of *in vivo* validated calpain-1 and −2 substrates). These cleavage events have been shown to promote motility of fibroblasts [31] and morphological changes to platelets to facilitate clotting [32]. It has also become apparent that calpain-1 and −2 modulate the activity of many substrates through limited proteolysis of regulatory domains. For example, calpain-2 mediated truncation of the androgen receptor results in ligand-independent activation in prostate cancer cells [33]. In normal cells, activation of calpain-1 and −2 can potentiate pro-apoptotic cell signaling upon Ca^{2+} influx induced by chemical insults or ER stress [2,34]. However, calpains are involved in both pro- and anti-apoptotic signaling pathways. *CAPNS1* gene knockout, which results in loss of both calpain-1 and −2, sensitized mouse embryonic fibroblasts to stimuli including staurosporine and tumor necrosis factor alpha, while conferring resistance to puromycin, camptothecin, etoposide, hydrogen peroxide, ultraviolet light and serum starvation [35]. These findings highlight the context-dependent and opposing pro- and anti-survival roles for calpain that might be exploited for therapeutic benefits. However, this potential opportunity has been limited by questions surrounding which calpain isoforms are playing which roles, and in what specific cellular contexts.

Calpains have been implicated in the progression of several human diseases. A large body of evidence suggests that calpain-2 dysregulation contributes to the neuropathogenesis of Alzheimer's disease, possibly through roles in amyloid beta aggregation and accumulation of tau neurofibrillary tangles (for a recent comprehensive review see [51]). Recent work by Baudry and colleagues suggests that calpain-1 and −2 may have opposing roles in neuronal cell protection and degeneration, respectively [52]. Consistent with this hypothesis, mutations in *CAPN1* have recently been linked with cerebellar ataxia [53]. Calpain-1 dysfunction has also been implicated in blood clotting disorders, with *CAPN1* knockout mice displaying reduced platelet aggregation and clot retraction [54].

Other calpains have also been implicated in other human diseases. Mutations in *CAPN3* result in limb-girdle muscular dystrophy type 2A, and this was phenocopied in mouse *CAPN3* knockout models [55] (reviewed in [56]). *CAPN5* mutations contribute to autoimmune uveitis, retinitis pigmentosa, and retinopathies that lead to irreversible blindness [57,58], while *CAPN14* is upregulated in response to IL-13 in esophageal epithelial cells in patients with eosinophilic esophagitis [59].

An emerging area of interest in the calpain field is their potential involvement in cancer, including regulation of cancer cell survival, metastasis, and susceptibility to chemotherapeutics and targeted agents. Activating or inactivating mutations in calpain genes do not appear to play a role in cancer. However, upregulation of calpain-1 and −2 have been described in several cancers (reviewed in [2]), and there is a growing appreciation for how calpain-1 and −2 are regulated by pro-oncogenic signaling pathways. These questions will be discussed in the following sections.

2.2. Evidence for calpain involvement in cancer

A growing number of translational studies suggest that aberrant calpain activity contributes to tumorigenesis and cancer progression (reviewed in [3–7].). An early clinical study reported that *CAPN1* transcript levels were associated with higher regional metastasis in renal cell carcinoma [60], while a more recent meta-analysis showed that high CAPNS1 protein expression independently predicted shorter overall survival across eight common cancer types, including colorectal, ovarian, lung, liver, and glioblastoma [61]. Similar studies suggesting pro-tumorigenic roles for calpain have emerged across

Table 1. Biologically relevant calpain substrates validated *in vivo*.

Substrate	Cleaved by	Type of Cancer*	Molecular/physiological context	References
Apoptosis Inducing Factor (AIF)	Calpain-1	Human non-small cell lung cancer	In U1810 cells, mitochondrial AIF is cleaved into a 57 kDa fragment that is subsequently released into the cytosol to promote apoptosis.	[101]
Androgen Receptor (AR)	Calpain-1	Prostate	In LNCaP cells, cleavage of AR produces ~80 kDa truncated fragment, contributing to prostate cancer cell survival.	[36]
Bax	Calpain-1/2	Breast, Leukemia	In Jurkat T and MCF-7 cells, Bax is cleaved into an 18 kDa fragment which promotes the release if cytochrome C and downstream activation of caspases.	[94]
Beclin-1	Calpain-1/2	Colorectal	In *Ras* mutant intestinal epithelial cells, calpain mediated degradation of beclin-1 promotes anchorage-independent growth.	[37]
β-catenin	Calpain-2	Breast, Prostate	N-terminal cleavage of β-catenin produces a 75kDa fragment that accumulates in the nucleus, promoting transcription of the Wnt signalling pathway.	[126]
BID	Calpain-2	Melanoma	Calpain cleaves BID into a 14kDa fragment in cisplatin-treated cells, resulting in an increase in cytochrome C release from the mitochondria.	[96]
Caspases	Calpain-1/2	Breast, Cervical, Neuroblastoma	Calpain-mediated cleavage directly activates caspases-3, 7, 10 and 12, and inhibits caspase-9.	[38,39]
CDK5	Calpain-2	Multiple	Calpain cleaves CDK5 cofactors p35 and p39 into p25 or p29 respectively, producing holoenzymes with longer half-lives, greater solubility and altered substrate specificity. Note that CDK5/p25 is also associated with neurodegenerative diseases.	[40,100]
Cortactin	Calpain-2	Breast	Cleaved between the actin binding repeats and the α-helical domain to promote invadopodium disassembly and cell migration.	[41,86,122,123]
CRMP-4	Calpain-2	Prostate	Cleaved into an N-terminal fragment, promoting migration and invasion via nuclear translocation and activation of DNA methyltransferase.	[65]
Cyclin E	Calpain-2	Breast	Cleaved into low molecular weight fragments that contribute to enhanced CDK activity, angiogenesis and metastasis.	[42,115]
E-Cadherin	Calpain-1/2	Breast, Prostate	Calpain cleaves within the cytoplasmic domain, preventing association of E-cadherin with β-catenin, γ-catenin and p120.	[125]
EGFR	Calpain-1	Breast	Calpain cleaves within cytoplasmic domain to downregulate kinase activity.	[111]
Ezrin	Calpain-1	Breast	First reported as a calpain substrate in gastric parietal cells, ezrin has also been implicated in focal adhesion dynamics and invadopodia turnover in MDA-MB -231 cells. Furthermore, ezrin regulates calpain-1 localization to promote cleavage of cortactin, FAK and talin.	[43,86]
FAK	Calpain-2	Breast, Colon	Cleavage to promotes focal adhesion turnover and cancer cell migration.	[86,87,120]
FLNA	Calpain-1/2	Melanoma, Prostate, Mouse Fibrosarcoma	Cleavage promotes increased nuclear localization of HIF1-α to promote tumor growth and angiogenesis.	[131,132]
HER2	Calpain-1	Breast	HER2 is cleaved in juxta- membrane region, cleavage products result in feedback inhibition of HER2, regulating trastuzumab sensitivity.	[111]
c-MYC	Calpain-1/2	Lymphoma, Prostate, Colon, Neuroblastoma	Cleaved into 'myc-nick,' promoting microtubule stabilization, autophagy, and drug resistance.	[104]
P53	Calpain-2	Breast, Cervical, Colon	Calpain cleavage facilitates p53 degradation and attenuation of apoptosis.	[44]
PP2A	Calpain-1/2	Breast	Calpain cleaves the B56 subunit of PP2A, preventing its association and subsequent deactivation of AKT.	[108]
PTP1B	Calpain-2	Breast	Cleavage releases PTP1B from the ER, thereby increasing its phosphatase activity and promoting invadopodium formation via Src.	[123]
Rb	Calpain-1	Cervical	Calpain cleavage results in degradation thereby alleviating repression of pro-tumorigenic E2F transcription factors.	[45,46]
RhoA	Calpain-1	Breast	Calpain mediated cleavage activates RhoA and inhibits cell spreading, promoting migration.	[47,124]
Spectrin	Calpain-1/2	Gastric, Lung, Leukemia	Well-established calpain substrate often used as an indicator of calpain activity. Cytoskeletal protein with roles in cell motility, apoptosis, platelet activation and long term potentiation.	[48,138]
Talin	Calpain-1/2	Multiple cancers	Calpain cleavage mediates cell adhesion and cytoskeletal remodelling to promote cancer cell migration.	[119]
Vinculin	Calpain-1/2	Multiple cancers	Cleavage promotes MT1-MMP membrane translocation and subsequent endothelial sprouting, supporting tumor angiogenesis.	[49]
C-fos/C-jun	Calpain-1/2	Mammalian cells (MEFs, NIH3T3)	PEST-containing transcription factors degraded by calpain, thereby regulating gene expression and apoptosis.	[35,99]
Dysferlin	Calpain-1/2	Muscular dystrophy	Cleaved into mini-dysferlin that promotes cell membrane repair.	[141,153]
NCS-1	Calpain-1	Chemotherapy induced peripheral neuropathy	Degradation results in loss of intracellular calcium signalling and irreversible damage to peripheral nerve fibers.	[83]
Src	Calpain-1	IschemicStroke, Neurodegenerative Diseases	Cleaved into a 52kDa truncated protein that facilitates neuronal cell death via AKT inactivation.	[50]

* Note: Type of cancer refers to the subtype(s) explored within the references provided. Many substrates/cleavage products have important biological functions in multiple cancer subtypes, as well as normal cell physiology. The last four entries are calpain substrates with established physiological relevance in non-cancer contexts.

various cancer subtypes. For example, high *CAPNS1* transcript expression was associated with metastasis and shorter survival in gastric cancer patients [62], while immunostaining has been used to show that colorectal cancer patients had poorer prognosis when tumors contained high levels of calpain-1 and low levels of its substrate filamin-A (FLNA) [63]. Interestingly, another study linked calpain-1 cleavage of FLNA to enhanced migration of androgen receptor-deficient prostate cancer cells [64]. Calpain-2 has been implicated in prostate cancer metastasis through regulation of gene expression by a mechanism involving cleavage of collapsin response mediator protein 4 (CRMP4), which in turn regulates DNA methyltransferase 1 expression [65]. In pancreatic cancer, a subtype infamous for rapidly spawning metastases, high tumor calpain-1 expression was associated with increased metastasis and shorter overall survival [66]. These authors showed that RNAi-based knockdown of calpain-1 in pancreatic cancer cell lines correlated with reduced proliferation and invasion *in vitro* [66]. The nonspecific calpain-1 and −2 inhibitor, calpeptin, suppressed pancreatic cancer cell proliferation, migration, and invasive behavior in *vitro* [67], but the *in vivo* effect of calpeptin in mouse xenograft models was dependent on co-engrafted fibroblasts [67], suggesting that calpain contributes to a stromal supporting role in tumorigenesis. Calpain-2 has also been identified as a potential predictive biomarker in ovarian cancer, with high expression associated with resistance to platinum-based therapies [68].

Multiple research groups have published observations of increased calpain expression in breast cancer. Storr and colleagues reported that elevated levels of calpain-1 and calpain-2 were associated with poor clinical outcomes in HER2-positive and triple negative breast cancer (TNBC) subtypes, respectively [69,70]. High calpain-1 expression was also associated with shorter disease-free survival in breast cancer patients treated with trastuzumab [71]. Conversely, low calpain-9 expression has been linked to poor outcomes in breast cancer patients who received endocrine therapy [72]. Interestingly, one study reported that high calpain-1 and high calpastatin levels were associated with better survival of patients with inflammatory breast cancer, while high calpain-2 and low calpastatin was correlated with improved survival in patients with non-inflammatory breast cancer treated with neoadjuvant chemotherapy [73], which invokes a more complex relationship between these calpain isoforms and their endogenous inhibitor in different types of cancer. Thus, while most of the current literature suggests a pro-tumorigenic role for calpains in cancer progression, there is conflicting evidence suggesting that, under some circumstances, calpains may have anti-tumorigenic roles. Higher calpain-1 and calpastatin levels in gastro-esophageal cancer predicted better survival in cohorts both with and without neoadjuvant chemotherapy [74]. In one study of pancreatic cancer subtypes, tumors of the bile ducts and ampulla were marginally more aggressive when calpain-1 and −2 levels were low [75]. In contrast, another pancreatic cancer study reported reduced survival for patients with increased calpain-1 expression [66], despite both these pancreatic cancer studies analyzing protein levels and employing similar immunohistochemistry approaches [66]. It has been observed that analyses of calpain protein levels and mRNA levels do not necessarily correlate. For example, in basal cell skin carcinoma, mRNA levels of *CAPN1* were significantly higher than in normal tissue while protein levels of CAPN1 were reduced [76]. These authors suggested that higher proteolytic and autolytic activity might be responsible for reduced calpain-1 protein, despite the increased presence of mRNA [76].

In addition to looking at expression of calpains, investigators have also looked at calpain substrates as prognostic biomarkers in cancer and other diseases. For example, FLNA has been characterized as a calpain-1 target, and its degradation along with elevated calpain-1 levels has been associated with poorer outcomes in colorectal cancer [63]. However, as with calpain itself, there is contrary evidence for the predictive power of FLNA. It was suggested that in glioblastoma, more FLNA cleavage predicts better patient survival and greater cancer cell apoptosis *in vitro* [77], presumably due to co-occurrence of pro-apoptotic calpain activation and FLNA degradation. Products of calpain breakdown are used as biomarkers for cell death in other diseases. For example, troponin I degradation products are an established clinical blood biomarker for injured myocardium in a variety of cardiovascular conditions [78], where it was shown that degradation is caused by hypoxia-induced calpain-mediated proteolysis [79]. Spectrin degradation products have been used as biomarkers for kidney disease and traumatic brain injury (TBI) [80]. Most recently, a calpain-2 mediated cleavage product of tyrosine phosphatase PTPN13, has been used as a biomarker for traumatic brain injury, where it is correlated with the severity of injury [81].

2.2.1. Calpains and chemotherapy-induced toxicities

In addition to their roles in disease progression, calpains have been implicated in cancer associated diseases linked to chemotherapy. Once again, calpains have been shown to have both detrimental and protective effects on patient health in the context of chemotherapy induced toxicities. For example, Peng and colleagues reported that overexpression of calpain-2 protected the heart against doxorubicin induced cardiotoxicity [82]. Calpain-2 is thought to promote AKT activation and subsequent upregulation of mitogen-activated protein kinase (MKP-1) to attenuate cardiomyocyte apoptosis in response to doxorubicin therapy [82]. Perhaps unsurprisingly, calpains are also involved in chemotherapy induced peripheral neuropathy. Treatment with the microtubule stabilizing drug, Taxol, has been shown to promote calpain-1 mediated cleavage of the neuronal Ca^{2+} sensor-1 (NCS-1) [83]. NCS-1 degradation was associated with a loss of intracellular Ca^{2+} signalling and irreversible damage to peripheral nerve fibers [83]. Similarly, calpain activation has been identified as an important early step in cisplatin-induced neurotoxicity [84]. Taken together, these observations underscore the potential of combining calpain inhibition with chemotherapy as a strategy to reduce cardiotoxicity and neurotoxicity [82–85].

2.3. Calcium signaling induces calpain activation in cancer cells

One model for calpain regulation predicts activation upon influx of extracellular Ca^{2+} into the cytoplasm via Ca^{2+}

channels in the plasma membrane. Substrate cleavage would be conditional upon cortical localization of calpain, its substrates, and a Ca^{2+} signal. A recent study in breast cancer cells showed that recruitment of calpain-1 to the plasma membrane by the ezrin adaptor protein promoted cleavage of its classical substrates talin, FAK, and cortactin [86]. Other studies show that induction of Ca^{2+} signals by various external treatments can trigger calpain-2 activation in cancer cells. For example, exposure of cancer cells to calcium lactate increased intracellular Ca^{2+} levels, induced calpain-2 mediated degradation of FAK and p53; and promoted cell motility [87]. Ca^{2+} can also be mobilized from the ER through activation of the inositol 1,4,5-trisphosphate receptor (IP3R). Such a mechanism was exploited to induce calpain-1-mediated apoptosis of an acute lymphoblastic leukemia cell line derived from a paediatric pre-B acute lymphoblastic leukemia patient [88]. Induction of Ca^{2+} influx and the ensuing calpain activation can feed into either pro-autophagy or pro-apoptosis signaling cascades, depending on other susceptibility factors, like intracellular levels of Atg5 or Bax, respectively [89]. While calpain inhibition on its own is often cytoprotective, when autophagy is induced under proteasomal stress, calpain inhibition can promote an anti-tumor effect of small-molecule cytotoxins [90].

2.4. Calpain mediated pathways in cancer

Calpain activation *in vivo*, limited by Ca^{2+} signals, is transient and localized to specific subcellular domains [86,91]. One of the challenges associated with identifying true physiologic substrates lies in the promiscuous activity of calpain outside of the cellular context. As a result, substrates identified *in vitro* remain hypothetical substrates that require validation in live cell systems. In this regard, the study of calpain is analogous to the study of protein kinase A (PKA) which can phosphorylate many substrates in biochemical assays with purified proteins [92]. However, its physiologic activity is closely regulated *in vivo* by localized cAMP signaling and the location of A kinase-anchoring proteins inside the cell [92]. Therefore, researchers must be careful when discerning between possible calpain substrates and biologically validated ones. Calpain substrates are found in many cancer-related signaling pathways and include products of oncogenes and tumor suppressor genes. We next discuss several key substrates involved in signaling pathways associated with cancer progression, metastasis, and treatment response. Please refer to Table 1 for a summary of several biologically relevant calpain substrates that have been validated in live cells.

2.4.1. Conventional calpain substrates associated with cell growth and death

For a comprehensive review of calpains in cancer apoptosis the reader is referred to the review by Storr and colleagues [2]. Briefly, several lines of evidence suggest calpains interact with the caspase family of cysteine proteases to initiate apoptosis [93]. Proteolytic cleavage mediated by conventional calpains directly activates caspases-7, −10 and −12 and inhibits caspase-9 [2]. Furthermore, calpain-1 and −2 mediated cleavage of the pro-apoptotic BCL2 family member, Bax, promotes the release of cytochrome c from the mitochondria which leads to downstream activation of executioner caspase-3 [94]. There is also evidence to suggest that Bax cleavage requires caspase-dependent activation of calpain [95]. As summarized in Table 1, other pro-apoptotic calpain substrates include BID [96,97], c-FOS [98,99], c-JUN [98,99], CDK5 [100], and apoptosis-inducing factor (AIF) [101]. Please refer to Figure 3 for examples of calpain substrates involved in cell survival and apoptosis.

In some contexts, calpains contribute to pro-survival pathways and may even promote resistance to anticancer therapies. Elucidating pro-survival roles for calpain has great relevance to cancer therapeutics, as researchers work to develop calpain inhibitors that may synergize with specific cytotoxic agents. For example, calpain-mediated cleavage promotes degradation of the p53 tumor suppressor, thereby attenuating apoptosis [102]. In human ovarian cancer cells, cleavage of p53-associated parkin-like cytoplasmic protein prevented nuclear localization of p53, thus inhibiting apoptosis [103]. Under stressful conditions, calpain cleaved cytoplasmic Myc to produce an N-terminally truncated protein termed 'Myc-nick' [104,105]. Myc-nick attenuated cell death by promoting drug resistance and autophagy under conditions of nutrient deprivation [104]. Grieve et al. demonstrated that *CAPNS1* knockdown was associated with increased sensitivity to the HSP90 inhibitor 17AAG in $HER2^{+}$ and TNBC cell lines [106]. This observation is believed to be the result of calpain-mediated effects on ABC transporters involved in drug efflux [106]. Calpain-2 has been shown to promote cancer cell survival via the PI3K-Akt-FoxO-$p27^{Kip1}$ signaling pathway [107]. Ho et al. demonstrated that calpain-2 knockdown was associated with reduced Akt phosphorylation, thus preventing the inhibition of Foxo3a mediated transcription of cyclin dependent kinase inhibitor p27 [107]. Bertoli and colleagues had also previously demonstrated that calpain negatively regulates Foxo3a by cleaving, thereby inactivating the Akt phosphatase, PP2A [108]. PP2A has also been shown to dephosphorylate calpain-1 and −2, attenuating their activation and reducing lung cancer cell migration and invasion *in vitro* [109].

Calpain mediated proteolysis of multiple members of receptor tyrosine kinase (RTK) family has been observed, where it can release the cytoplasmic domain from the membrane. For example, calpain cleaves Met, producing a stable and pro-metastatic p45 fragment [110]. Calpain-mediated cleavage in the juxta-membrane region of HER2 was also reported, which produced either the complete cytoplasmic domain or a truncated fragment [111]. In that context, inhibiting calpain resulted in accumulation of more full-size RTK and greater cell survival [111]. In line with such a model, MacLeod et al. have shown that knockout of calpain-1 and −2 in an activated HER2-driven transgenic mouse mammary carcinoma model changed its phosphoproteomic landscape, producing more phospho-EGFR [112].

While calpains can target multiple members of RTK superfamily, alterations in RTK signaling have been implicated in the regulation of calpain as well. For example, overexpression of

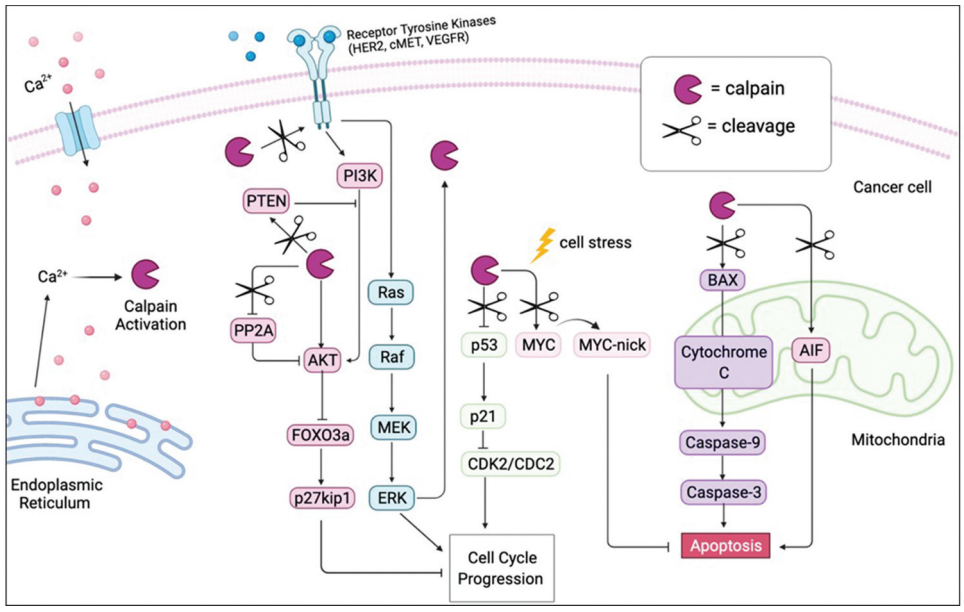

Figure 3. **Calpain substrates in cell survival and apoptosis**. Calpains become activated by increases in intracellular Ca^{2+} concentration, through Ca^{2+} influx or release from intracellular stores. Calpains are implicated in many key signalling pathways associated with cell death and survival including the PI3K-AKT [107,108], ERK [113], p53 [102], MYC [104,105], caspase [38,39] and AIF pathways [101]. Created with BioRender.com.

HER2 induced *CAST* transcription, which correlated with higher levels of Src, FAK, and ERK – all predicted calpain substrates [113]. Another study also suggested EGF-mediated activation of calpain as a mechanism of cyclin G2 degradation [114]. Cyclin E [115] and cyclin D1 [116] are also suspected calpain substrates. In addition, calpain activation has been observed as an effector of VEGF stimulation in endothelial cells, promoting angiogenesis [117]. Miyazaki et al. reported that overexpression of calpastatin in mouse endothelial cells attenuated angiogenesis by preventing calpain-mediated cleavage of SOCS3 and downstream activation of the JAK/STAT pathway [118]. Calpain involvement in tumor angiogenesis remains to be fully elucidated, but evidence including that described above supports pro-metastatic roles for calpain.

2.4.2. Conventional calpains and metastasis

Calpains contribute to tumor cell migration, invasion, and metastasis by altering focal adhesion dynamics and promoting cytoskeletal remodelling (reviewed in [2,3,5–7]). A graphical overview of some of these processes and calpain-1 and −2 functions are illustrated in Figure 4. Huttenlocher and colleagues demonstrated that calpain-2 mediated proteolysis of the

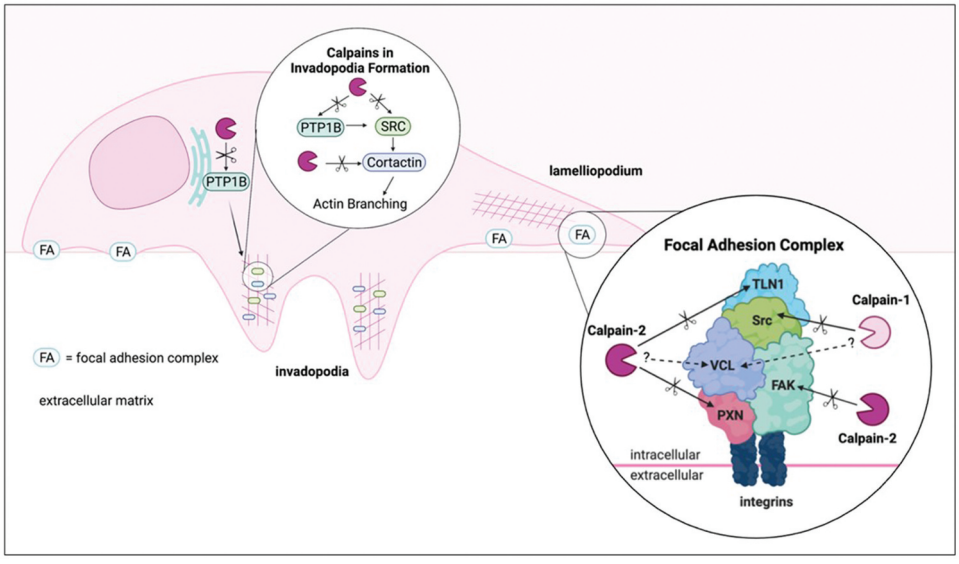

Figure 4. **A schematic representation of calpain substrates in cell motility**. Calpain cleaves PTP1B to facilitate its relocalization from the ER to the cytosol. There, PTP1B de-represses Src which subsequently activates cortactin and promotes actin branching – a key event in the formation of invadopodia structures. Calpain-1 cleaves Src kinase [50], and calpain-2 cleaves paxillin (PXN), focal adhesion kinase (FAK) and talin (TLN1) [127]. Vinculin (VCL) is a calpain substrate, but isoform specificity is unknown [128]. Colocalization of PXN, FAK, VCL and TLN1 at FAs and their structural organization are demonstrated [129]. Src is also localized at FAs [130].Created with BioRender.com

cytoskeletal protein talin is required for adhesion disassembly [119]. Furthermore, focal adhesion kinase (FAK) is cleaved by calpain-2 *in vivo*, promoting adhesion turnover, in part by altering talin dynamics [120]. A more recent study found that silencing *CAPNS1* in renal carcinoma cells reduced talin cleavage and significantly impaired migration and invasion *in vitro* [121]. Calpain-2 mediated cleavage of the actin-assembly protein cortactin also promoted cell migration by modulating invadopodium formation [122]. The actin-membrane linker, ezrin, has been identified as a substrate of calpain-1, but interestingly, ezrin also acts upstream of calpain to regulate focal adhesion and invadopodia turnover [86]. Protein tyrosine phosphatase 1B (PTP1B) becomes hyperactivated in response to calpain-2 mediated truncation [123]. Interestingly, this cleavage removes a C-terminal ER localization domain that allows PTP1B to relocalize from the ER to the cytosol, where it acts as a positive regulator of the nonreceptor tyrosine kinase c-Src to promote invadopodium formation in metastatic breast cancer cells [123]. The calpain-1 isoform has been shown to negatively regulate cell adhesion by cleaving and inactivating the RhoA GTPase, a key player in the formation of stress fibers and focal adhesion complexes [124]. Calpains also cleave the cell-cell adhesion molecule, E-cadherin, preventing its association with beta-catenin, gamma-catenin, and p120 catenin [125]. The functional inactivation of E-cadherin has been shown to promote a metastatic phenotype through loss of epithelial cell-cell adhesion [125]. Calpain has also been shown to cleave and activate beta-catenin in metastatic prostate and breast cancer cells, thereby promoting Wnt pathway activation [126].

Calpain-mediated cleavage of the actin cross-linking protein FLNA also contributes to metastatic behaviour. Salimi and colleagues showed that the migratory behavior of human melanoma cells depends on a 90kDa FLNA fragment produced by calpain proteolysis [131]. Calpain-mediated FLNA cleavage was also implicated in hypoxic response and tumor angiogenesis by promoting the nuclear localization of HIF-1α [132]. FLNA has also been shown to regulate the transcriptional activity of androgen receptor (AR) by sequestering the FHL2 coactivator [133]. Calpain cleavage of both FLNA and AR promotes association and nuclear localization of a FHL2/AR complex [133]. However, there are conflicting data pertaining to whether calpain-cleaved AR is activated in a ligand-independent fashion or degraded [134,135]. In an animal model of prostate cancer, where the calpain-AR interaction is presumed to play a significant role, inhibition of calpain-2 resulted in less invasive cancer cells [136].

Calpains also regulate membrane plasticity and protrusions through cleavage of the relevant scaffolding proteins. Dysregulation of Ca^{2+} homeostasis in cancer cells promotes aberrant calpain activation resulting in cleavage of cytoskeletal components [137]. Spectrin, a cortical scaffolding protein, is a well-established calpain substrate, and it has frequently been used as an indicator of calpain activation *in situ* [138]. A recent study showed a novel pathway for inducing calpain-mediated cleavage of spectrin through DCC, a netrin-1 receptor [91]. Decreased cortical presence of spectrin through calpain cleavage was shown to increase the biogenesis of extracellular vesicles (EVs) [139], which act as paracrine effectors of malignant cells. EV production is upregulated in cancer cells and contributes to metastatic and drug resistant phenotypes [109].

Calpain-1 and −2 expression was also associated with membrane 'blebbing' in mouse embryonic fibroblasts, which correlated with altered protein levels of Rho GDP-dissociation inhibitor and cofilin-1 [140]. Calpain-cleavage of dysferlin has also been linked with membrane repair of mechanical damage by promoting vesicle-membrane fusion [141]. Together, these observations implicate calpain in disassembly of the cortical actin cytoskeleton to allow greater membrane fluidity and membrane alterations.

2.4.3. Conventional calpains and immune response

There is considerable evidence suggesting involvement of calpains in immune signaling. For example, calpain can cleave interleukin-1 alpha to produce its mature form [142]; however, caspase activation may be necessary for complete activation and release of IL-1 alpha, even after calpain cleavage [143]. Calpain has also been implicated in promoting inflammatory signaling of NF-kB by cleaving the IkB regulatory protein [144]. Dysregulated expression of *CAPN14* has also been associated with eosinophilic esophagitis [59] Another study found that inhibiting calpain activation promoted autophagic degradation of PD-L1, which was associated with beneficial anti-tumor effects in an animal model. Beclin-1, a calpain substrate, is a suspected mediator of this process [145]. Many other roles for calpain in regulation of inflammation have recently been reviewed [146]. While many of these studies indicate calpain inhibition could protect against tissue and organ damage associated with excessive or chronic inflammation, there are also observations suggesting calpain inhibition could be detrimental [146]; thus, careful study of potential side effects will need to be carried out in future clinical trials of calpain inhibitors.

2.5. Calpains as therapeutic targets

There are numerous readily available active site directed calpain inhibitors. However, there is considerable debate about the specificity of these inhibitors. Many that are described as calpain-specific inhibitors (for example, ALLN or calpeptin) exhibit inhibitory effects on other proteases, including cathepsins, the proteasome, or caspases (as disclosed in major vendor' specifications). Peptidomimetic active-site-directed inhibitors have been developed based on the primary sequence of calpastatin. While more specific, these peptides generally lack good cell permeability and pharmacokinetic properties; however, a recent review of calpain inhibitors cites a patent on a blood-brain barrier permeable peptidomimetic inhibitor [147]. The cell membrane or blood-brain barrier permeability is achieved by linking the CAST-mimetic sequence to lipid-soluble compounds, such as cholesterol, or to a cell-penetrating peptide sequence, such as from penetratin [147,148]. The benefit of such solubilized peptide inhibitors over conventional active site directed cysteine protease inhibitors of calpains is their high specificity, with 4–6 orders of magnitude difference in K_i between inhibiting calpains versus cathepsins, the proteasome, or caspases[149].

Allosteric inhibitors of calpain exist as well. The most prominent of them, PD150606 and AMG853, are presumed to bind hydrophobic pockets in the PEF domains [150]. Despite published models and co-crystal structures showing such interactions, Low et al. showed that, at least in case of PD150606, the mode of action is not through binding to a hydrophobic groove, and that PD150606 is a much weaker inhibitor compared to classic protease inhibitors [151]. Currently, there are still no calpain inhibitors approved for clinical use.

2.5.1. Possible side-effects of calpain inhibition from preclinical studies

Systemic inhibition of calpain-1 or calpain-2 is likely to have distinct physiologic effects. The phenotype of calpain-1 deficient (*CAPN1* mutant) mice, dogs, and humans is a predisposition to cerebellar ataxia and muscle wasting [53]. Additionally, *CAPN1* knockout mice have a defect in platelet aggregation and clot retraction, but surprisingly, there is no significant bleeding defect [54]. There is evidence for several roles of calpain in platelet homeostasis [152]. Muscle-specific deficiency in both calpain-1 and calpain-2 (through *CAPNS1* knockout) caused dystrophy in aged mice, linked to the known role for calpain-1 and −2 in myoferlin cleavage during Ca^{2+}-induced membrane repair [153]. Complete germline calpain-2 deficiency in mice was associated with embryonic lethality [154,155]; however, ubiquitous inducible or tissue-specific *CAPNS1* knockout (resulting in deficiency in both calpain-1 and −2) in adult mice is well tolerated. There are no known human pedigrees with loss-of-function mutations in *CAPN2* or *CAPNS1*, which would likely be embryonic lethal in the homozygous state. However, there are multiple studies in human pedigrees describing loss-of-function mutations of *CAPN1* that are predicted to compromise functions in PC1, PC2, CBSW and PEF(L) domains [156,157]. Inhibition of both calpain-1 and −2 in the brain affects long-term potentiation and susceptibility to traumatic brain injury [52] and protects against cytotoxic neuronal cell death [158]. Interestingly, inhibition of calpain-2 alone appears to be beneficial for neuronal survival [52]. Similarly, indirect inhibition of calpains was also beneficial to survival of neuronal cells in a model of Alzheimer's disease, where calpain activity was reduced through pharmacological inhibition of histone deacetylase and transcriptional upregulation of calpastatin [159]. There is no evidence that transient calpain inhibition would have long-term detrimental effects outside of the traumatic brain injury scenario. In contrast, preclinical animal models suggest that calpain inhibition may protect against neuropathy induced by cancer chemotherapeutics [84].

Other calpain isoforms with well-established roles in human disease are calpain-3, −5 and −14. Hypomorphic mutations in *CAPN3* are associated with limb-girdle muscular dystrophy, observed both in human pedigrees and in mouse knockout models [56], hypermorphic mutations of *CAPN5* lead to retinopathy [58], and CAPN14 is dynamically upregulated in patients with eosinophilic esophagitis [59]. Thus, inhibition of calpain-3 could cause muscular dystrophy, while patients with mutations in *CAPN5* or *CAPN14* could benefit from inhibitors.

These data suggest that intermittent systemic inhibition of calpain-1 and −2 might be well tolerated, but considerations should be made for toxicities towards embryonic development, or possibly, effects of extended exposure to calpain inhibition on selected tissues. As of this writing, calpain inhibitor BLD-2660 is undergoing a clinical trial for treatment of COVID-19 to reduce tissue IL-6 and prevent lung fibrosis. Another phase-2 trial of this drug in idiopathic pulmonary fibrosis was recently withdrawn.

2.5.2. Other proteases in cancer

Calpains are not the only class of proteases studied in the context of cancer biology and therapy. Matrix metalloproteases degrade extracellular matrix (ECM) to promote cell motility (reviewed in [160]). The proteasome recycles intracellular proteins, which promotes cell survival of both normal and malignant cells (reviewed in [161]). Cathepsins, classically lysosomal enzymes, also participate in MHC-mediated antigen presentation, ECM degradation for cell invasion, and pro-survival autophagy [162]. The plasmin protease of fibrin in blood clots can activate latent MMPs, and its activators and inhibitors have been implicated in cancer invasion [163]; and caspases, general-purpose pro-apoptotic factors, can be hindered by dysregulation in calpain-mediated proteolysis [2].

Perhaps the most studied among these, with promising selective anti-tumor effects in cancer models, are inhibitors of proteasomal proteases [161] and matrix metalloproteases (MMP) [160]; but both types are excessively vulnerable to acquired resistance and relapse in clinical settings, especially in solid tumors. However, in specific cases of myeloma and mantle-cell lymphoma, proteasome inhibitors like bortezomib, ixazomib and carfilzomib have been approved even as a first-line therapy [161]. Failures of proteasome inhibitors in cancer have been traced to acquired mutations in the drug-binding pockets of the enzyme or upregulation of heat-shock and antioxidant response pathways [161]. Combination of proteasome and HSP90 inhibitors might overcome such resistance, but there are no clinical data on the benefits of such combinations available yet [161]. MMP inhibitors, which have similarly failed to produce reliable anti-cancer effects and also had a multitude of adverse side-effects, were speculated to be not sufficiently isoform-specific or requiring a very early intervention before the tumor has become invasive [160]. Some side effects of proteasome or MMP inhibitors are diarrhea, thrombocytopenia, and painful neuropathy or dyspnea, musculoskeletal syndrome, and transaminitis, respectively [160,164].

In contrast, transient inhibition of calpain-1 and −2 are not expected to produce severe side effects in adults. While active-site inhibition of these calpains would also likely affect other calpain isoforms and other structurally homologous cysteine proteases, the dimeric nature of calpain-1 and −2 allows the possibility of allosteric protein-protein interaction inhibition of the PEF domains, which would likely have little off-target effect due to a limited number of other proteins containing PEF domains and due to relatively low sequence homology among them.

3. Conclusions

In summary, our growing understanding of the calpain system suggests that targeting calpain proteases represents a promising approach for the treatment of a wide range of diseases, including cancers. As we learn more about calpain biology, the complexity of calpain functions may reveal opportunities that can be exploited for therapeutic benefits. As novel calpain substrates continue to emerge, our understanding of the impact of calpain proteolysis of these substrates on pathways that they participate in may provide rationale for therapeutic strategies consisting of calpain inhibitors combined with other targeted agents. The development of isoform specific pharmacologic calpain inhibitors may be required to allow this approach to expand further into animal models, and eventually human trials.

4. Expert opinion

Calpain proteases have been studied for nearly fifty years, yet the extent to which we understand their biologic functions is still frustratingly incomplete. While they are widely conserved, studies of calpains in simpler organisms have provided limited insight into their developmental or physiologic roles. Extensive biochemical and structural studies have given us a solid understanding of their proteolytic activities and regulation by Ca^{2+}, calpastain and other mechanisms, and crystal structures are available for some isoforms. Structure-function and genetic studies in cell systems, humans, and mice have also revealed relationships between mutations and disease phenotypes for some isoforms; and these studies continue to provide more detailed insights into their cellular and physiologic functions. However, we still do not have a good understanding of the cellular context under which the various calpain isoforms become activated, what controls their selection of specific substrates, and how substrate cleavage affects the global cell signaling network in normal biology and disease. Nevertheless, evidence of dysregulated calpain expression in diseases including cancers, fibrosis, muscular dystrophy, retinopathy, eosinophilic esophagitis, and Alzheimer's has provided incentive to better understand potential etiologic roles for calpains in these diseases and to develop inhibitors that may be used therapeutically. In this regard, a challenging and exciting area of calpain research is the identification of physiologic substrates and elucidation of how calpain-mediated proteolytic cleavage affects their functions. Over one hundred protein substrates have been reported, and this list continues to grow. However, detailed mechanistic information regarding how calpain cleavage affects substrate functions and cellular behaviors is lacking for most of these substrates. The application of TAILS-MS or other emerging proteomic methods to quantify calpain-mediated cleavage of substrates under specific conditions has the potential to identify additional substrates and better understand calpain roles in cell signaling, as well as the implications for systemic calpain inhibition in disease contexts.

We are particularly interested in substrates that play roles in the regulation of cancer cell migration, invasion, survival, and proliferation because of the importance of these cell behaviours in metastasis and tumor progression. Emerging studies provide rationale for inhibiting calpains to interfere with these mechanisms as a strategy to suppress tumorigenesis and make cancer cells more susceptible to other therapeutics, including radiation, chemotherapies and targeted agents. Some of this insight comes from correlative translational studies that link high calpain-1 and −2 expression with poorer prognosis in different types of cancer. Preclinical studies using cultured cancer cells, engraftment, and transgenic mouse models are emerging that show genetic disruption of calpain-1 and −2 is associated with suppressed tumor growth or reduced metastatic behaviors. Beneficial effects of calpain-1 and −2 genetic disruption have also been seen in models of fibrosis. While these observations inspire efforts to develop more effective and specific calpain inhibitors, these efforts have not yet resulted in approved therapeutics. This represents an important unmet need which holds promise for the treatment of several diseases.

Recent efforts to inhibit calpains include the development of calpastatin-derived peptidomimetics and *in silico*-informed molecular design to exploit the hydrophobic pocket of CAPNS1. However, we still lack potent selective pharmacological inhibitors for *in vivo* studies and specific tools for assessing calpain activity *in situ*. These challenges need to be addressed using gene-specific knock-out model systems to verify that calpain is the selective target of experimental inhibitors. Another weakness is our incomplete understanding of calpain isoform-specific functions in different biological contexts, including cancers. For example, our current understanding is that calpain-1 and calpain-2 have both redundant and isoform-specific roles in terms of cleavable substrates and affected pathways; and current data suggests that disruption of either isoform can suppress tumorigenesis in some models. These observations argue that targeting either calpain-1 or calpain-2 has therapeutic potential in cancer. Not only does calpain inhibition have the potential to render cancer cells more susceptible to specific therapeutic challenges, but there is evidence that systemic calpain inhibition could also protect normal cells and tissues against the cytotoxic effects of some cancer treatments. This underscores the need to more fully elucidate the cell-specific roles that calpain isoforms are playing, especially in the context of systemic cancer therapies, and to ultimately develop isoform selective calpain inhibitors to use in rationally designed combinatory cancer therapies. The recent discovery that loss of function mutations in *CAPN1* are associated with ataxia, and studies showing a protective effect of calpain-1 in brain injury suggest that calpain-2 specific inhibitors would be preferable in the treatment of neurodegenerative diseases and cancer.

Our understanding of calpain continues to grow and much of the historical and recently emerging knowledge supports the idea that isoform specific calpain inhibitors will become effective therapeutics in cancers and other diseases. The challenges going forward include improving our understanding of the effects of calpain inhibition on different cell types in various disease contexts, refining our knowledge of the structure and regulation of different calpain isoforms, developing biomarkers that reveal *in vivo* calpain activity, and using that

knowledge to develop isoform-specific inhibitors that will be safe and effective therapeutics.

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