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Calpains as a Target for Idiopathic Pulmonary Fibrosis?

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ABSTRACT

The following review provides a preliminary assessment of CAPN9 as a possible target for idiopathic pulmonary fibrosis (IPF). IPF is defined as a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause, primarily occurring in older adults, limited to the lungs, and associated with the histopathologic and/or radiologic pattern of usual interstitial pneumonia (UIP). The calpains are a group of calcium-dependent, intracellular, non-lysosomal cysteine proteases. Calpains are thought to be associated primarily with cytoskeletal function and many gene products associated with carcinogenesis are substrates of calpain family enzymes. Calpains need to be strictly regulated since they catalyse irreversible processing in the cell. It has been hypothesised that calpains localize to the plasma membrane under activating conditions in order to be located where they can respond to brief calcium influxes from the opening of calcium channels, resulting in localized and transient activity. Under the hypothesis that fibrosis is driven by epithelial-mesenchymal transition (EMT), several groups have reported on the role of cytokines such as IL-1, IL-6, TNFα and TGFβ in triggering myofibroblast differentiation and subsequent fibrosis and the possible role of calpains. CAPN9 is an unprecedented molecular target and as such, its validity can only be ascertained based on human clinical data. Like other proteases, CAPN9 should lend itself to structure-based design approaches and allow the creation of ligand selectivity against *e.g.* cathepsins and caspases; additional work is however required to clarify whether selectivity towards other calpains is desired or necessary. In addition, a better understanding of whether the calpain system has an obligate role in fibrosis would be helpful in de-risking discovery and development efforts.

1. INTRODUCTION

The following review provides a preliminary assessment of CAPN9 as a possible target for idiopathic pulmonary fibrosis (IPF).

IPF is defined as a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause, primarily occurring in older adults, limited to the lungs, and associated with the histopathologic and/or radiologic pattern of usual interstitial pneumonia (UIP) [1]. From a pathophysiological point of view, the current hypothesis is that IPF is an epithelial-fibroblastic disease, in which unknown endogenous or exogenous stimuli disrupt the homeostasis of alveolar epithelial cells, resulting in diffuse epithelial cell activation and aberrant epithelial cell repair [2]. The condition has a poor prognosis, and beyond lung transplantation, the only drug approved specifically to treat IPF is Pirfenidone (Intermune-Roche), which appears to provide a small survival benefit along with improvements in FVC in patients with mild-moderate IPF [3,4].

2. CAPN9 – EXPRESSION, STRUCTURE, FUNCTION AND REGULATION

The calpains are a group of calcium-dependent, intracellular, non-lysosomal cysteine proteases. The calpain proteins are heterodimers consisting of an invariant small (28 kDa) subunit and variable large (80 kDa) subunits. The large subunit possesses a cysteine protease domain, and both subunits possess calcium-binding domains. The calpains are found in both plants and vertebrates and 14 isoforms have been found in mammals [5, 6]. Calpains are thought to be associated primarily with cytoskeletal function and many gene products associated with carcinogenesis are substrates of calpain family enzymes, including FOS, JUN, p53, and ESR1 [6].

Expression.

While the two most characterised members CAPN1 and CAPN2 (“micro-“ and “millicalpains”, respectively) are ubiquitously expressed, other calpains show sharply defined tissue specificity; for example, CAPN3 is essentially only expressed in skeletal muscle, CAPN6 in placenta and CAPN11 in testis.

Lee *et al.* [7] obtained a cDNA encoding CAPN9, which they termed nCL-4 (novel calpain large subunit-4). Northern blot analysis revealed CAPN9 expression predominantly in stomach and small intestine, with low levels in other organs. Western blot analysis detected

expression of an 80 kDa protein in transiently transfected cells. Using differential display of normal and cancerous gastric tissue, Yoshikawa *et al.* [8] identified cDNAs encoding a splice variant of CAPN9, which they termed GC36. As compared to CAPN9, GC36 has a ser292-to-arg substitution and lacks the subsequent 26 amino acids. Northern blot analysis revealed expression of approximately 2.6- and 2.0-kb transcripts of CAPN9 and GC36 in normal but not gastric cancer cell lines. RT-PCR analysis detected down-regulated expression in gastric cancer tissues and cells.

Structure

Sequence analysis shows that the 108 kDa, 690-amino acid human CAPN9 protein, which is 86% identical to the rat protein, has the four typical calpain domains. The protease domain (domain II) is the most conserved, followed by domain III and the calcium-binding region in domain IV, which has 5 EF-hand structures [7]. The crystal structure of CAPN9 has been solved [9]; the structure of CAPN9 indicates that auto-inhibition in this enzyme is mediated through large intra-domain movements that misalign the catalytic triad (see below).

Function

Calpains convert intracellular calcium signals [10] into a proteolytic signal by catalysing limited cleavage of target proteins [5, 11]. Like all cysteine proteases, CAPN9 acts *via* a catalytic triad in which the first step is deprotonation of a thiol in the active site by an adjacent amino acid with a basic side chain, usually histidine residue. The next step is nucleophilic attack by the deprotonated cysteine's anionic sulphur on the substrate carbonyl carbon. In this step, a fragment of the substrate is released with an amine terminus, the histidine residue in the protease is restored to its deprotonated form, and a thioester intermediate linking the new carboxy-terminus of the substrate to the cysteine thiol is formed. The thioester bond is subsequently hydrolysed to generate a carboxylic acid moiety on the remaining substrate fragment, while regenerating the free enzyme. Among the known cellular substrates are cytoskeletal proteins, as well as some receptors and integral membrane proteins like the Na^{+}/Ca^{2+} exchanger NCX-3 [12].

The cleavage specificity of calpains appears to be weakly dictated by the primary sequence of its protein substrates. Rather, other structural factors within the substrate, such as the necessity for an unstructured backbone region or possibly a specific three-dimensional conformation, appear to be more important in defining the precise cleavage sites [13-15].

The physiological role of the calpains in general, and CAPN9 in particular is poorly understood; calpains primarily appear to play roles in cytoskeletal homeostasis (motility, the cell cycle machinery, apoptosis and in mediating necrosis). Given its specific tissue expression profile under normal conditions, it is thought that CAPN9 has a physiological role in the gastrointestinal tract [5, 6].

Regulation

Calpains need to be strictly regulated since they catalyse irreversible processing in the cell. It has been hypothesised that calpains localize to the plasma membrane under activating conditions [16, 17] in order to be located where they can respond to brief calcium influxes from the opening of calcium channels, resulting in localized and transient activity. Deactivation of calpain can come about in several ways: binding to the endogenous calpain inhibitor calpastatin [18]; autoproteolytic inactivation or simply the dissipation of local high calcium levels.

Calpastatin is the only known endogenous specific inhibitor of the calpains [19]. Among calpain homologues so far examined, calpastatin inhibits CAPN1, CAPN2, CAPN8 [20] and CAPN9 [21] but not CAPN3 [22]. Calpastatin is proteolysed by CAPN3, implying that CAPN3 helps regulate conventional calpains in skeletal muscle [22]. One calpastatin molecule contains four inhibitor units; each unit inhibits one calpain molecule with variable efficiency [23-25]. Oligopeptides as short as 20 amino acids derived from these inhibitory units can inhibit calpain, although with reduced efficacy. Calpastatin has poor primary sequence conservation between species, despite its high specificity even to different species' conventional calpains, which are highly conserved: humans and rat CAPN1 are 89% identical, whereas rat and human calpastatin are only 66% identical. The three-dimensional structure of CAPN2 co-crystallized with a calpastatin fragment and Ca^{2+} revealed that the intrinsically unstructured properties of calpastatin enable it to bind calpain tightly while looping several adjacent amino acid residues out from the active site to protect itself from proteolysis [26,27].

3. ROLE OF CALPAINS IN FIBROSIS

Under the hypothesis that fibrosis is driven by epithelial-mesenchymal transition (EMT), several groups have reported on the role of cytokines such as IL-1, IL-6, TNF□ and TGF□

[27-33] in triggering myofibroblast differentiation and subsequent fibrosis and the possible role of calpains.

Derbel *et al.* [34] studied possible causes for the pancreatic fibrosis and diabetes seen in many patients with cystic fibrosis and conducted analyses of the role of polymorphisms in six genes. Using oral glucose tolerance tests, Derbel *et al.* subdivided 163 adult pancreatic insufficient CF patients by glucose tolerance and found evidence for the association between CF-related diabetes and UCSNP-19 polymorphism in the calpain-10 gene, leading to the conclusion that UCSNP-19 of CAPN10 may be involved in the pathogenesis of diabetes in CF.

Li *et al.* [35] investigated whether disruption of calpain-4 would reduce myocardial hypertrophy and fibrosis in a streptozotocin (STZ) mouse model of type 1 diabetes. The function of calpains was studied both by cardiomyocyte-specific CAPN4 knockout and by use of calpastatin transgenic mice. Calpain activity, cardiomyocyte cross-sectional areas, and myocardial collagen deposition were significantly increased in STZ-induced diabetic hearts, accompanied by elevated expression of hypertrophic and fibrotic collagen genes. Deficiency of CAPN4 or overexpression of calpastatin reduced myocardial hypertrophy and fibrosis this diabetic model, leading to the improvement of myocardial function. Letavernier *et al.* [36] reviewed other studies suggesting that calpains are involved in transduction processes leading to myocardial remodelling, fibrosis and heart failure.

Tabata *et al.* [37] noted that calpains are found in relatively high concentrations in synovial fluids in patients with rheumatoid arthritis, which is known to be triggered by IL-1, IL-6, TNF□ and TGF□[38,39] and hypothesised that inhibition of calpains could be a viable path also to address pulmonary fibrosis. Tabata *et al.* examined the protective effects of the non-selective peptidomimetic calpain inhibitor calpeptin on bleomycin-induced pulmonary fibrosis in C57Bl/6 mice. Calpeptin histologically ameliorated bleomycin-induced pulmonary fibrosis in mice and decreased the expression of IL-6, TGF□ , angiopoietin-1 and collagen type I□$_1$ mRNA in mouse lung tissues. *In vitro,* calpeptin reduced production of IL-6, TGF□ , angiopoietin-1 and collagen synthesis from lung fibroblasts and both IL-6-dependent proliferation and angiopoietin-1-dependent cell migration.

Ma *et al.* [40] examined the role of calpains in pulmonary vascular remodelling in two rodent models of pulmonary hypertension, demonstrating that attenuated calpain activity in calpain-knockout mice or rats treated with a calpain inhibitor resulted in prevention of increased right

ventricular systolic pressure, right ventricular hypertrophy, as well as collagen deposition and thickening of pulmonary arterioles in models of hypoxia- and monocrotaline-induced pulmonary hypertension. Additionally, inhibition of calpain *in vitro* blocked intracellular activation of TGF-β1, which led to attenuated Smad 2/3 phosphorylation and collagen synthesis. Finally, smooth muscle cells of pulmonary arterioles from patients with pulmonary arterial hypertension showed higher levels of calpain activation and intracellular active TGF-β.

Li *et al.* [41] used conditional knockout (ERC re$^{+/-}$ CAPN1$^{flox/flox}$) mice and primary human lung fibroblasts (HLFs) to investigate the relationship between calpain and TGFβ1. CAPN1 KO mice were protected from fibrotic effects of bleomycin; in separate experiments, bleomycin also induced increases in TGF-β1 *via* calpain activation in HLFs. Moreover, TGFβ1 also activated calpain. Li *et al.* hypothesised that this crosstalk between calpain activation and TGFβ1 triggered the downstream signalling pathway including TGFβ1, Smad 2/3, and non-Smad (Akt) pathways, as well as collagen-I synthesis in pulmonary fibrosis.

As the above examples show, literature provides gene expressions, cellular and functional evidence for the involvement of several calpains in TGF□-induced EMT and fibrosis across a series of model systems including primary human tissue. While many of these studies suggest an important role for calpains, they cannot be considered to demonstrate an obligate role (*i.e.* observed results could be a co-variate of some unrelated process). The study by Li *et al.* [41] is, however, compelling in indicating a TGFβ-mediated causative role of CAPN1 in pulmonary fibrosis.

Beyond the work by Kim and co-workers [42], little has been published specifically regarding the possible role of CAPN9 in fibrosis; most work around CAPN9 has focussed on its role as a tumour suppressor and in ischaemic processes in the CNS (see below).

4. CALPAINS AND CANCER

As noted above, many gene products associated with carcinogenesis are substrates of calpains, including products of oncogenes and tumour suppressor genes such as c-fos, c-jun, p53, pp60src, the oestrogen receptor and the adhesion molecule integrin. Neurofibromatosis type 2 (NF2) protein, a tumour suppressor implicated in schwannomas and meningiomas, is calpain-sensitive [43]. Calpains have also been implicated in various aspects of carcinogenesis, including cell-cycle progression, cellular differentiation, and apoptosis [44,

45]. CAPN1 expression is correlated with increased malignancy in renal cell carcinoma [46] and in breast cancer, activities of calpain were significantly higher compared with those of normal breast tissues, and were higher in the ER-positive tumours than in ER-negative ones [47]. In contrast, Davis *et al.* [48] investigated the expression of CAPN9 in a large cohort of early-stage breast cancer patients (n = 783) using immunohistochemistry. Patients had long-term follow-up information available for analysis. Their study showed that low expression of CAPN9 was associated with patients over 40 years of age ($p = 0.025$), smaller tumour size ($p = 0.001$), lower tumour stage ($p = 0.009$), a more favourable Nottingham Prognostic Index value ($p = 0.002$) and positive oestrogen receptor status ($p = 0.014$). CAPN9 expression was not correlated to overall survival in this patient cohort, however low CAP9 expression was associated with adverse survival in patients who received endocrine therapy ($p = 0.033$), which remained significant in multivariate Cox regression analysis accounting for potential confounding factors (hazard ratio (HR) 0.56, 95% confidence interval (95% CI) = 0.36-0.89, $p = 0.013$). Low CAPN9 expression was also associated with adverse survival in patients with an intermediate Nottingham Prognostic Index value ($p = 0.009$), and remained so in multivariate analysis (HR = 0.54, 95% CI = 0.36-0.82, $p = 0.003$).

The already mentioned study by Yoshikawa *et al.* [8] showed that CAPN9 expression was downregulated in gastric cancer tissues and cell lines of both differentiated and poorly differentiated type. Independently, depletion of CAPN9 by antisense RNA resulted in the cellular transformation of and tumorigenesis by murine NIH 3T3 fibroblasts [49]. These results suggest that CAPN9 might be a type of tumour suppressor. Hata *et al.* [50] analysed $CAPN8^{-/-}$ and $CAP9^{-/-}$ mice, neither of which strains showed any gross developmental abnormalities and had normal gastric mucosae. Both mice strains were, however, susceptible to gastric mucosal injury induced by ethanol administration. Both strains showed significant decreases in stomach CAPN8 and CAPN9.

5. CALPAINS AND THE CNS

Although of limited relevance to fibrosis and pharmacological calpain inhibition, the *activation* of calpains has implications in the CNS, with suspected links to Alzheimer's and Parkinson's diseases, ischaemia and stroke and at least one hereditary disease (limb-girdle muscular dystrophy Type 2, LGMD2A) is known to be caused by mutations in the gene encoding for CAPN3 [51]. In all these cases, a common mechanism is activation *via* increased intracellular calcium; in cerebral ischemia and traumatic brain injury, decreased

blood flow to affected brain areas results in increases in presynaptic vesicular glutamate release and inhibition of glutamate re-uptake by adjacent astrocytes. The resultant excessive build-up of glutamate activates ionotropic glutamate receptors (NMDA, AMPA and kainate receptors) in the postsynaptic membrane and sustained influx of Na^+ and Ca^{2+} through these receptors. Upon membrane depolarization, voltage-gated neuronal Ca^{2+} channels open, enabling Ca^{2+} influx and subsequent activation of CAPN1 and 2 [44,52]. In Alzheimer's disease, chronic toxicity of aggregated amyloid β peptides (Aβ1–40, Aβ1–42) together with build-up of glutamate leads to sustained intracellular calcium elevation in susceptible central neurons and therefore CAPN1 and CAPN2 activation [53,54].

6. KNOWN CALPAIN INHIBITORS

Aside from the endogenous calpain inhibitor calpastatin (see above), several small molecules aldehyde-containing peptidomimetic inhibitors have been described:

– MDL-28170 (benzyl-*N*-[(2S)-3-methyl-1-oxo-1-[(1-oxo-3-phenylpropan-2-yl) amino] butan-2-yl] carbamate) is a non-selective calpain inhibitor, with a K_i value of 10nM, but also inhibits and cathepsin B with a K_i value of 25 nM [55]. It is a peptidomimetic similar to calpeptin and leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-argininal), and like calpeptin and leupeptin, comprises an aldehyde function.

– Calpeptin (benzyl-*N*-[4-methyl-1-oxo-1-(1-oxohexan-2-ylamino) pentan-2-yl] carbamate) is a CAPN1 inhibitor with an IC_{50} of 5 nM [37].

– SJA-6017 (*N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal) with an IC_{50} of 80 nM [56].

– E-64 ((*1S,2S*)-2-(((*S*)-1-((4-guanidinobutyl)amino)-4-methyl-1-oxopentan-2-yl)-carbamoyl)-cyclopropanecarboxylic acid, an epoxide-containing naturally occurring peptide

– AKT275 (Z-Leu-Abu-ethyl amide) is a non-specific calpain inhibitor with an IC_{50} >1 mM.

In addition, three non-peptidic compounds without aldehyde functions have been described:

– PD 150606 ((*Z*)-3-(4-iodophenyl)-2-sulfanylprop-2-enoic acid) inhibits CAPN1 and 2 with K_i values of 0.21 and 0.37 μM, respectively [57].

– ABT-705253 (*N*-(1-carbamoyl-1-oxohex-1-yl)-2-[E-2-(4-dimethylaminomethylphenyl)-ethen-1-yl]benzamide) is an orally bioavailable benzoylalanine [58].

– SNJ-1945 (((*1S*)-1((((*1S*)-1-benzyl-3-cyclopropylamino-2, 3-di-oxopropyl) amino) carbonyl)-3-methylbutyl) carbamic acid 5-methoxy-3-oxapentyl ester [59].

No selective CAPN9 inhibitors have been described in the open literature or in published patents.

7. CAPN9 AS A MOLECULAR TARGET – A CRITICAL ASSESSMENT

Obligate Role?

The calpain family has been subject to interest as possible therapeutic targets since the mid-1990's for a range of pathologies and major Pharma organisations (Pfizer, Abbot) have had ongoing programmes to identify small-molecule inhibitors.

The calpains appear to show low and overlapping substrate specificity and their action appears to be regulated primarily by differential tissue expression. In most cases, the endogenous substrates are not known. So far, existing peptidic and non-peptidic inhibitors have shown low isoform selectivity, making assessment of differential function difficult.

The knock-out models highlight that the calpains may have a causative role in the development of fibrosis following TGF□ challenge, but data also suggest significant redundancy and no experimental system shows complete rescue. Whether this redundancy is caused by functional overlap with other calpains (or indeed other cysteine proteases such as cathepsins) or to complexity in upstream signalling is difficult to assess; a possible bias in existing literature could be the focus on TGF□ as the triggering factor, while the *in vivo* situation likely involves a more complex situation, with several other profibrotic factors such as IL-1, IL-6 and TNF□ .

In this context, a comparison to pirfenidone is compulsory; although no definite mechanism of action for pirfenidone has been established, it is likely that it inhibits both production and activity of TGFβ. In a bleomycin-induced lung injury hamster model, pirfenidone significantly suppressed TGFβ gene transcription by 33% [17] and overexpression of procollagen I and III genes [18]. In animal models of hepatic fibrosis, pirfenidone also decreased TGFβ and collagen type 1 mRNA expression [19]. In addition, pirfenidone inhibits pulmonary fibroblast expression of HSP 47, a collagen-specific molecular chaperone thought to play an important role in extracellular matrix synthesis and remodelling [20]. In cultured renal cortical fibroblasts isolated from rats with ureteric obstruction, pirfenidone has been shown to inhibit fibroblast mitogenesis and expression of connective tissue growth factor [21]. Various animal models of fibrosis demonstrate that pirfenidone also exerts anti-

inflammatory effects. It suppresses bleomycin-induced increased pulmonary vascular permeability and increased neutrophil- and macrophage influx [22]. In a superantigen-induced shock model, mice treated with pirfenidone had substantially improved survival, with the outcome closely correlating with a reduction in TNFα and interleukin-1 expression [23].

No data regarding the effects of pirfenidone on the calpain system have appeared in the literature, but it is noteworthy that the magnitude of the treatment effects of non-selective calpain inhibitors are comparable to pirfenidone in the bleomycin-induced mouse model [37, 40, 60]. An interesting question is whether selective CAPN9 inhibition is superior to a pan-calpain approach; these data are in stark contrast to the data from Ma *et al.* [40] and Li *et al.* [41].

Drug Design Aspects

The active site of any enzyme serves the dual purpose of binding substrates and performing catalysis. For proteases, improvements in the selectivity constant (k_{cat}/K_m) for small substrates are mainly driven through improvements in K_m. Optimized cleavage sequences are therefore strongly linked to an optimized binding and are valuable for the design of sensitive and specific peptidomimetic substrates, as well for the design of lead compounds in drug and inhibitor design [62,63].

Intrinsically, proteases must be considered tractable targets, given a large number of protease inhibitors marketed for various indications. Previous data from the peptidomimetics demonstrate that it is relatively straightforward to achieve nanomolar inhibitors, and the availability of high-quality X-ray structures should aid in designing appropriately potent non-peptidic inhibitors. Cysteine protease inhibitors belong to two general classes: irreversible, covalent suicide inhibitors and reversible (or allosteric) inhibitors:

1. The most widely explored irreversible inhibitors use an electrophile to modify the active cysteine covalently and a recognition motif for binding to the active site. Commonly used electrophilic groups include epoxysuccinates, vinyl sulfones, allyl sulfones, vinyl sulfonates, diazomethyl ketones and fluoro- or chloromethyl ketones [64-66]. Other examples include vinyl ketones, vinyl esters, and vinyl sulphones, which provide alternate Michael acceptor electrophiles [67, 68]. There has been a tendency to avoid irreversible, suicide inhibitors in drug development on account of perceived risks with hapten formation, non-specific,

modification of off-target proteins and technical issues in characterising drug metabolism, although a large number of approved suicide inhibitor products suggests that these issues can be managed [69,70].

2. Reversible cysteine protease inhibitors comprise aldehydes and ketones such as MDL-28170 and calpeptin and hemiacetals such as SJA-6017 and a series of a-keto derivatives such as ABT-705523 [58]. Nitriles have also been used and an example is odanacatib (Merck), which is a cathepsin K inhibitor in Phase III studies for osteoporosis [71]. Allosteric inhibitors have also been described and include PD-150606 and similar compounds [57, 72].

In the case of CAPN9, and based on their physiological roles, the key selectivity challenges are likely to be cathepsins and possibly also caspases. Whether selectivity towards other calpains can (or should) be achieved is difficult to assess based on available data.

Possible Intrinsic Safety Concerns

The data on CAPN9 regarding tumorigenicity are relatively weak and conflicting; the much-cited paper by Yoshikawa *et al.* [8] reports on decreased gene expression levels in gastric cancer cell lines and as such cannot be considered to constitute sufficient evidence of a suppressor role for CAPN9 in gastric cancer. Similarly, the study by Hata *et al.* [50] only discusses ethanol-related insults to the gastric mucosa and provides no evidence for neoplastic transformation as a result of CAPN9 knock-out. The study by Davis *et al.* [48] is more interesting, but suggests that CAPN9 status might be a covariate rather than a predictor for outcome. The data from Liu *et al.* [49] regarding the neoplastic transformation of 3T3fibroblasts are however relevant and would merit further attention as part of the validation of CAPN9 as a therapeutic target.

9. OVERALL CONCLUSIONS

CAPN9 is an unprecedented molecular target and as such, its validity can only be ascertained based on human clinical data. Like other proteases, CAPN9 should lend itself to structure-based design approaches and allow the creation of ligand selectivity against *e.g.* cathepsins and caspases; additional work is however required to clarify whether selectivity towards other calpains is desired or necessary. In addition, a better understanding of whether the calpain system has an obligate role in fibrosis would be helpful in de-risking discovery and development efforts.

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