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Sajal Chakraborti
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Proteases in Health and Disease

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Preface

The idea for the book arose on 17th February, 2012 at the "Cardiology Research Convergence" meeting held in New Delhi. We (Dhalla and Chakraborti) met in the pleasant morning at the lounge in the venue at the *All India Institute of Medical Sciences* and discussed about the research on different aspects of proteases. During our discussion, we agreed to jointly edit two books on proteases: (1) Proteases in Health and Disease and (2) Role of Proteases in Cellular Dysfunctions for the series "Advances in Biochemistry in Health and Disease" (Editor: Prof. N.S. Dhalla; Springer Publisher, New York). Accordingly, we embarked on the project associating colleagues and friends to contribute to the 22 chapters for the book entitled "Proteases in Health and Disease". Each contributor has been a key scientific player in raising new knowledge on the role of proteases. We want to express our most sincere gratitude to all the authors for their scholarly contributions and the time and energy they spend in this endeavour. We are indebted to Dr. Vijayan Elimban and Mrs. Eva Little (St. Boniface Hospital Research, Winnipeg, Canada), Ms. Melanie Tucker and Ms. Rita Beck (Springer USA, New York) for their time and effort in bringing this book as scheduled. We are also thankful to Prof. Dilip Kumar Mohanta (Vice Chancellor, University of Kalyani, Kalyani, WB, India) and Dr. Naren L. Banik (Department of Neurosciences, Medical University of South Carolina, SC, USA) for their encouragement.

Proteases form one of the largest and most diverse families of enzymes known. Once considered primarily as "enzymes of digestion", it is now clear that proteases are involved in every aspect of organism function. Members of the families including serine, cysteine, threonine, glutamic, metallo- and aspartic proteases act to promote cellular proteolysis. With the advent of whole genome sequencing, this classification system has expanded by the necessity to encompass the diverse catalytic repertoire found in nature, and any of their deregulation may cause derangement of cellular signaling network resulting in different pathophysiological conditions such as tumor progression, vascular remodeling, atherosclerotic plaque progression, ulcer and rheumatoid arthritis. Consistent with these essential roles of proteases in cell behavior and survival and death of all organisms, many infected

microorganisms also require proteases for replication or use proteases as virulence factors, which have facilitated the development of protease-targeted therapies for a variety of parasitic diseases.

Proteases are being recognized more and more as important players in a wide range of biological processes; for example, the cell cycle, blood clotting, angiogenesis, apoptosis, cell differentiation and growth, cell motility, lipid metabolism, antigen presentation and cell fate determination are all dependent upon the controlled action of proteases. When the activity of proteases is not regulated appropriately, disease processes can result, as is seen in Alzheimer's disease, cancer metastasis and tumor progression, inflammation, pain, atherosclerosis and hemophilia.

The book entitled "Proteases in Health and Disease" represents a comprehensive overview of the multifaceted field of protease in cellular environment and focuses on the recently elucidated functions of complex proteolytic systems in physiology and pathophysiology. The proteases treated include cysteine proteases such as μ- and m-calpains; matrix metalloproteases such as MMP-2, MMP-9 and ADAMS; serine proteases in leishmania; and aspartic proteases in fungus and malaria parasite. The chapters address the role of proteases in cancer cell invasion, bone diseases, lung diseases and infectious diseases, dealing the basic biochemistry behind the disease states as well as therapeutic strategies based on protease inhibition. With this multidisciplinary scope, this book bridges the gap between fundamental research and biomedical and pharmaceutical applications, making this a thought-provoking reading for basic and applied scientists engaged in biomedical research.

This book is an outcome of enthusiasm of various renowned experts in the relevant research areas and contains two subdivisions: Part I, Molecular and Biochemical Aspects of Proteases; and Part II, Involvement of Proteases in Diseases Processes. In Part I, the chapters illustrated some of the major proteases such as calpains, matrix metalloproteases, fibrinolytic serine protease and aspartic proteases, which play a significant role in a variety of pathologies and could be target for therapy either by their up-regulation or down-regulation. On the other hand, Part II deals with functional roles of the individual proteases in the progression of diseases such as cardiovascular and inflammatory lung disease, malaria, cholera, autism spectrum disorder, hepatitis and ischemia reperfusion injury-induced cardiac diseases. Each chapter in this book raises many questions that need to be addressed for finding appropriate solutions in the area of pathophysiology and therapy of diverse diseases.

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Part I
Molecular and Biochemical Aspects of Proteases

An Overview of Endoplasmic Reticulum Calpain System

Krishna Samanta, Pulak Kar, Tapati Chakraborti, and Sajal Chakraborti

Abstract Calpains, a family of Ca^{2+}-dependent cysteine proteases, can modulate their substrates structure and function through limited proteolytic activity. Calpain mediated proteolysis of intracellular proteins is a key step in various cellular processes such as cytoskeleton modulation, cell migration, cell cycle progression and apoptosis. Calpain activity is controlled *in vivo* by calpastatin, a multiheaded endogenous polypeptide encoded by the calpastatin gene that specifically inhibits calpain. Calpains have previously been considered as the cytoplasmic enzymes; however, recent research have demonstrated that m-calpain and calpastatin are present in endoplasmic reticulum and play important roles in a variety of pathophysiological conditions including necrotic and apoptotic cell death phenomena. This review summarizes function and regulation of the endoplasmic reticulum calpain system, focusing on the relevance of its roles in several cellular and biochemical events under normal and some pathophysiological conditions.

Keywords Endoplasmic reticulum • Calcium • m-calpain • Calpastatin • Apoptosis

1 Introduction

Since the first description of calpain (calcium-activated neutral protease) in 1964 by Guroff [1], extensive progress has been made regarding the identity, structure, activity, localization, and physiological and pathological functions of calpains. To date, the

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number of identified mammalian calpain protease family members has grown to 14 [2]. Based on their tissue expression patterns, calpains are classified as ubiquitous or tissue specific [3]. Two ubiquitous isoforms, μ- and m-calpains and their endogenous inhibitor, calpastatin have been identified and studied extensively. The interaction of calpastatin with calpain prevents both activation and catalytic activity of calpain [4]. *In vitro* studies have shown that calpain and calpain fragments bind to calpastatin and that calpastatin fragments bind to calpain in the presence of Ca^{2+} [5].

Intracellular Ca^{2+} is a critical signal transduction element in regulating pulmonary smooth muscle contraction, proliferation as well as expression of a variety of gene responsible for pulmonary hypertension [6]. Cytosolic Ca^{2+} dynamics is regulated, at least partly, by the ER, which takes up when cytosolic Ca^{2+} levels are high and releases it when cytosolic Ca^{2+} levels are low [7]. The endoplasmic reticulum (ER) is one of the largest sub-organelles in eukaryotic cells. In the ER, the essential function of membrane proteins, membrane lipids and secreted proteins synthesis, calcium storage, folding and maturation of protein take place [8]. So the efficient functioning of the ER is essential for most cellular activities and survival. Therefore, tight regulation and maintenance of ER homeostasis is vital for normal cellular functions. The ER lumen and its membrane contain a set of resident proteins that are involved in ER function [9]. Immunological studies have shown that in some cell types, the major calpain isozyme, m-calpain and calpastatin are not only present in the cytoplasm, but also present in sub-organelles, for example, ER [10]. A variety of agents increase intracellular free Ca^{2+} levels, which in turn up regulates Ca^{2+} dependent protease including calpain. Several calcium regulatory proteins in ER play important roles in regulating $[Ca^{2+}]_i$ dynamics [11]. Therefore, calcium-dependent cleavage of these calcium regulatory proteins by m-calpain in the ER, as evidenced from different studies, appears to be an important mechanism for intracellular Ca^{2+} overload, which could be important for the manifestation of several cellular dysfunctions such as, pulmonary hypertension, edema and ischemia reperfusion and cell death [12–14]. Thus, it has now been established that endoplasmic reticulum calpain-mediated cell death is linked to several pathophysiological events of various diseases [12–14].

The present review comprises overview of the endoplasmic reticulum calpain system and its activation and regulation mechanisms. Additionally, the physiological and pathophysiological roles of endoplasmic reticulum have been explored.

2 Overview of the Calpain System

Calpain system is known to be comprised of three molecules: two Ca^{2+}-dependent proteases, μ- and m-calpains, and their endogenous inhibitor calpastatin [15]. The calpain family is conserved in many different species, from fungi to human [15]. Some calpains are ubiquitously expressed-calpains 1, 2, 4, 5, 7 and 10 [15, 16], whereas others are found in specific tissues: calpain 3 (skeletal muscle) [17], calpain 6 (placenta) [18], calpain 8 (smooth muscle) [19], calpain 9 (stomach) [19],

calpain 11 (testes) [20, 21], calpain 12 (skin after birth) [22] and calpain 13 (testes and lung) [20]. Additionally, calpains are divided into two groups based on domain IV structure [15]. Typical calpains (1, 2, 3, 8, 9, 11, 12, and 14) contain a penta-EF hand in domain IV that can bind Ca^{2+}, the calpain small subunit (only calpains 1, 2 and 9 have been shown to dimerize) or calpastatin. Atypical calpains (5, 6, 7, 10, 13 and 15) lack a penta-EF hand in domain IV and are unable to bind the calpain small subunit or calpastatin [23–25] (Fig. 1a, b).

Most calpains contain four structural domains Domain I of calpains 1, 2 and 9, are autolysed upon Ca^{2+} stimulation [15], but whether other typical calpains undergo autolysis of domain I is not clear. In atypical calpains, domain I is not cleaved and the function of domain 1 is unknown for many of these calpains, except for calpain 10 which contains a mitochondrial targeting sequence [26]. Domain II contains the active site with the catalytic triad of cysteine, asparagine and histidine, and this catalytic triad is conserved throughout the entire family, except for calpain 6 that lacks proteolytic activity [27]. In addition to containing the catalytic triad, crystallographic studies revealed that domain II can bind two atoms of Ca^{2+} and assist in calpain activation [23, 28]. Domain III contains two Ca^{2+}-binding sites and a phospholipid binding motif in the C2-like area [29]. These Ca^{2+} and phospholipid binding residues are conserved throughout the family, except for calpain 10 [23]. Also, domain III is believed to regulate calpain activity through specific electrostatic interactions and is involved in substrate recognition [23, 30]. Domain IV contains the penta-EF hand that can bind Ca^{2+}, calpastatin or the small subunit [15]. These penta-EF hands are thought to be one of the most important features in calpain activation [3]. Calpain small subunit (28 kDa) contains two domains, domain V and VI and the only known function for domain V is to bind to the C-terminus region of domain IV in large calpain subunits [31]. Domain VI is identical to domain IV in that it has a penta-EF hand that is available for Ca^{2+}-binding and heterodimer formation [32].

Atypical calpains 5, 6 and 10 have the same general structure as typical calpains for the first three domains, but lack the penta-EF hand in domain IV [23, 33]. (Fig. 1b). Instead, atypical calpains contain a divergent domain IV, which presently has an unknown function. Because there is no penta-EF hand, which binds the majority of the Ca^{2+}, likely many atypical calpains either do not require large Ca^{2+} concentrations or their activity is only modulated by Ca^{2+} [26]. Such differences in domain IV suggest different activation and inhibition patterns for atypical calpains.

The full-length calpastatin has six domains: XL, L, I, II, III and IV [15, 34] (Fig. 1c). Domains I–IV are essential for calpain inhibition and each of them consists of three subdomains- A, B and C, with subdomain B playing a central role in calpain inhibition. A 27 residue peptide (CS), containing most of subdomain B from domain I of human calpastatin, is a potent and specific inhibitor of calpain [25]. (Fig. 1d). Of the four domains, the order of inhibition effectiveness is: I > IV > III > II [35]. Little is known about the XL domain other than the three protein kinase A phosphorylation sites [36] and the function of the L domain is still unclear. Because calpastatin must bind domain II and domains IV or VI to inhibit calpains, it seems unlikely that atypical calpains are inhibited. Therefore, atypical calpains must have other regulatory mechanisms Immunolocalization studies show that the calpain and

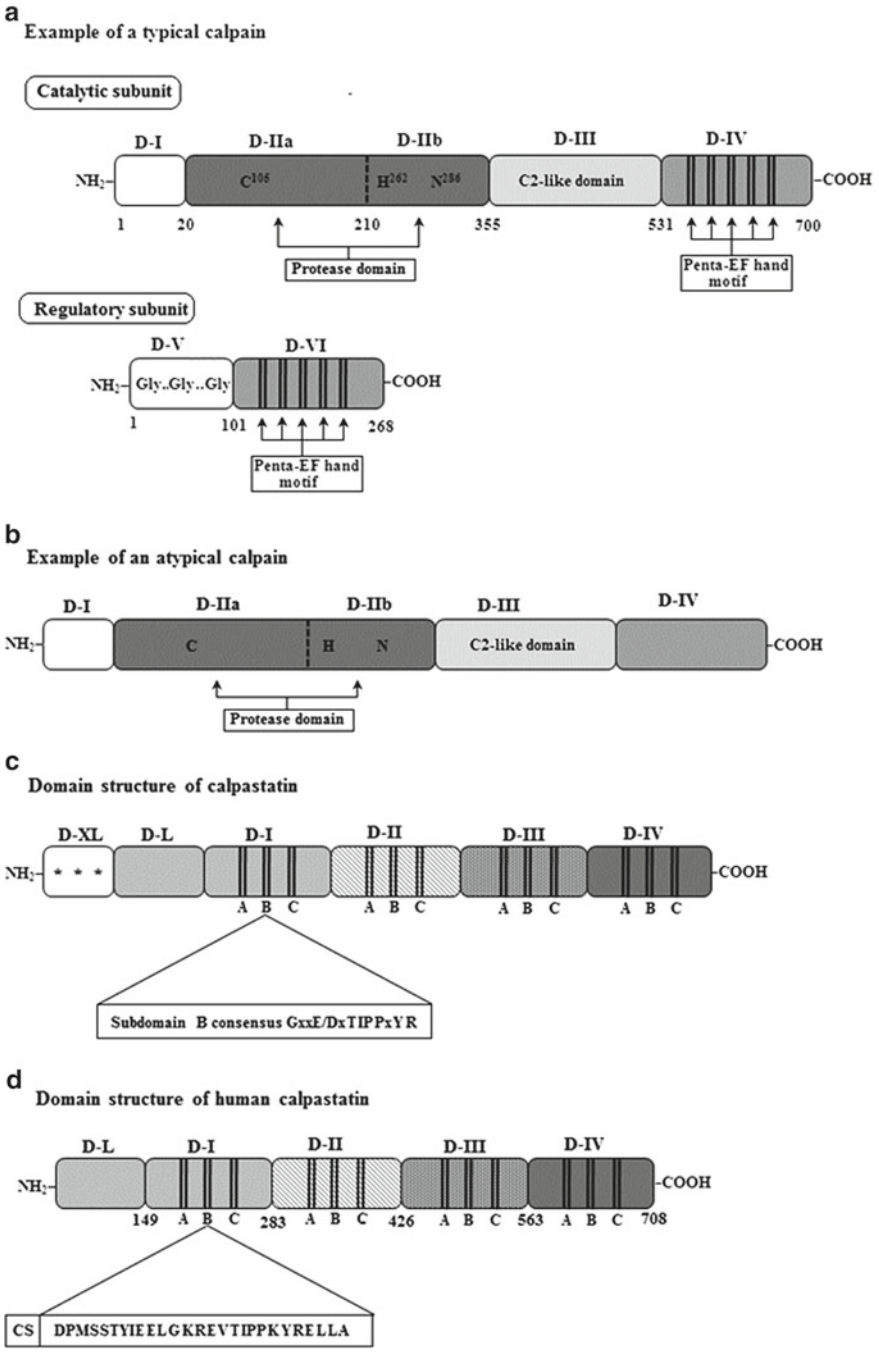

Fig. 1 Schematic diagram of calpains and calpastatin molecules. (**a**) Schematic representation of domain architecture of the typical calpains. The catalytic subunit contains domains I–IV Domain I

calpastatin are generally localized in the same areas in cells [37], and calpastatin binds to and inhibits the calpain in the presence of Ca^{2+} [36]. Regulation of the calpain-calpastatin interaction is, therefore, appears to be important in controlling calpain activity.

3 Endoplasmic Reticulum Calpain System and Its Regulation

Although, calpains are considered to be cytoplasmic enzymes, recent research have shown that calpains also exist in several suborganelles in the cell. Immunolocalization studies have shown that both μ-calpain and m-calpain and calpastatin are located exclusively intracellularly; the few reports of extracellular localization have been attributed to poor fixation procedures or to diseased tissues where the calpains/calpastatin had "leaked" from cells [38]. Most immunolocalization and immunoprecipitation studies have found that the calpain and calpastatin are colocalized in cells [10, 38, 39]. Recent research demonstrated that calpains are also present in several subcellular organelles such as, caveolae vesicles of the plasma membrane, mitochondria, endoplasmic reticulum, Golgi apparatus and nucleus [40–44]. In this article, we intend to focus only the calpain system present in the endoplasmic reticulum and how they are regulated by their endogenous regulatory proteins in the ER. Recent research by Samanta *et al* [42] demonstrated that m-calpain (MCp) containing 80 kDa large and 28 kDa small subunits are present in the ER membrane; whereas only 80 kDa m-calpain (LCp) is present in the lumen of the ER. It has also been reported that although calpastatin molecules having molecular weight of 110 and 70 kDa are present in the ER membrane [42], but no calpastatin molecule is present in the ER lumen. This group has also purified and characterized MCp, LCp and calpastatin from the ER and established a brief comparative study between MCp and LCp Samanta *et al* [45] reported that although ER MCp is almost similar with cytosolic m-calpain in molecular mass, optimum pH and Ca^{2+} concentration, isoelectric

Fig. 1 (continued) contain residues 1–19 and interact with domain VI of the small subunits. Domain II is divided into two subdomains, IIa and IIb, which carry residues of Cys 105, His 262, and Asn 286 responsible for calpain catalytic activity Domain III harbors the C2 area and binds with phospholipids Domain. IV is the C-terminal end of the large subunit. It consists of five consecutive EF-hand motifs. The regulatory subunit contains domain V, which is a highly flexible, glycine-reach region, and domain VI, which is a Ca^{2+} binding region, similar to domain IV of the catalytic subunit (**b**). Domain architecture of an atypical calpain, which has no regulatory subunit and penta EF-hand motifs like typical calpain (**c**). Diagram of the domain structure of calpastatin with a consensus sequence for subdomain B * represents PKA phosphorylation site (**d**). Schematic diagram showing the domain structure of human calpastatin A, B and C regions are subdomain having significant sequence homology with each domain. Subdomain B contains a highly conserved 27 oligopeptide (calpastatin peptide, CS) and responsible for calpain inhibition

point, kinetic parameters (Km, kcat and kcat/Km values), autolysis and CD spectra, but differs in its state of phosphorylation [45, 46]. On the other hand, although LCp is similar with cytosolic m-calpain in optimum pH and Ca^{2+} concentration, isoelectric point, kinetic parameters (Km, kcat and kcat/Km values) and CD spectra, state of phosphorylation; but differs in molecular mass, autolysis and regulation by interacting with ERp57 [45, 46]. Therefore, ER MCp and LCp are similar in optimum pH and Ca^{2+} concentration for their maximal activities, isoelectric point, kinetic parameters (Km, kcat and kcat/Km values) and CD spectra; but differ in molecular mass, state of phosphorylation, autolysis and their regulations and functional activities MCp, LCp and calpastatins of ER are closely related to the corresponding enzyme (m-calpain) and its endogenous inhibitor calpastatin molecule from other species as evidenced by physico-chemical, kinetic and functional properties and also by N-terminal amino acid sequence analyses. Another group has also reported the presence of full length 110 kDa m-calpain (80 kDa large with 28 kDa small subunits) presents in the ER membrane but only large subunit of m-calpain (80 kDa) is present in the lumen of the ER [43, 47]. Although cDNA of m-calpain does not reveal any known signal sequence to target it to the ER [15], a number of potential mechanisms can be offered. For instance, m-calpain contains C2-like domain acidic loops [29, 30, 48] similar to the C2 domain acidic loop of phospholipase A_2 [49]. The binding of Ca^{2+} to the C2 domain acidic loops of phospholipase A_2 targets this enzyme to the ER [49]. In a similar fashion, m-calpain may also be targeted to the ER Structurally, the regulatory subunit is composed of two domains, domain-V and domain-VI Domain-VI contains an EF-hand pentamer and thus is capable to associate with membrane through these hydrophobic EF-hands [50]. Domain-V of the regulatory subunit is unusual in that it contains 40 glycine residues that are moderately hydrophobic, 30 hydrophobic residues, 5 prolines, and 26 polar residues [15]. It could be envisioned that the polyglycine repeats within the regulatory subunit domain-V confer a high degree of flexibility to the molecule, whereas the hydrophobic residues allow for association between the regulatory subunit and membranes. Therefore, binding of the regulatory subunit to ER membrane would provide a platform for m-calpain binding to the ER through hydrophobic interactions between EF hands of m-calpain domain-IV and regulatory subunit domain-VI.

The ability of calpastatin to associate with the ER may be effected by the position of the positively charged amino acids: lys-lys-arg-his-lys-lys in the calpastatin L-domain [15]. This stretch of amino acids would effectively tether calpastatin to the negatively charged phospholipids head groups on the cytosolic side embedded to the ER membrane.

In a similar way 80 kDa m-calpain is present within the lumen of the ER despite absence of any known N-terminal signal peptide capable of targeting in the ER lumen [51]. So, the entry of this molecule into the lumen of the ER could occur by some other means. One probability is that the 80 kDa m-calpain possesses an internal topogenic signal sequence that assembles post translationally through intramolecular conformational changes, and it is this new topology that targets m-calpain into the ER lumen [47]. A number of proteins utilize internal rather than N-terminal signal sequences to enter the ER lumen [52, 53]. To resolve how 80 kDa m-calpain gains

entry into the ER lumen will require deletion or substitution mutations to identify an internal topogenic signal sequence [47]. Whereas, the functional significance of the 80 kDa m-calpain activity residing in the lumen of the ER remains to be established, a number of advantages are imparted to the cell, for example, this may provide an opportunity for 80 kDa m-calpain to cleave substrates shortly after their synthesis and insertion into the ER membrane as well as those that accumulate in the ER lumen. Another potential role for intraluminal 80 kDa m-calpain may be to aid in the removal of misfolded proteins and also regulate the Ca^{2+} regulatory protein [47, 54].

The presence of calpastatin molecules has been reported in the ER membrane but not in the ER lumen [42, 47]. Therefore, the regulation of endogenous calpain activity in the ER is important and interesting. Many of the tissues that contain calpain have also been reported to contain calpastatin [55]. In some cases calpastatin is absent, wherein calpain is regulated by an another regulatory molecule, ERp57 [56, 57]. ERp57 mediated regulation of mitochondrial μ-calpain has been reported [57]. ERp57 (also known as ER-60, ERp60, ERp61, GRP58) is a member of protein-disulfide isomerase (PDI) family [57, 58]. It is mainly present in the lumen of the ER and can also be found in the nucleus, extracellular space, cytosol and cell surface [58]. ERp57 functions mainly in the refolding of the large subunit of calpain, which is involved in the formation of disulfide bonds to form functional conformations and also in prevention of proteolytic degradation of calpain [57]. It has now been established that association between MCp and calpastatin occurs in the cytosolic face of the ER membrane; whereas LCp and ERp57 are associated in the ER lumen [42]. Thus endogenous ER calpains are regulated mainly by its endogenous inhibitor, calpastatin as well as ERp57 and such localization and association of calpain in the ER gives the opportunity of calpain to act on several calpain substrates in the ER, which could cause the degradation of several ER proteins and regulates the ER function in several physiological and pathophysiological conditions.

4 ER, Calpain and Cellular Ca^{2+} Homeostasis

ER is involved in virtually every aspect of cellular physiology [59]. ER plays a vital role in many cellular processes, including Ca^{2+} storage and release, lipid and protein synthesis, folding and post-translational modification [60]. ER is also involved in cellular signaling and organelle–organelle communication, including ER stress-dependent activation of transcriptional processes, Ca^{2+} signaling and communication to the plasma membrane Ca^{2+} channels and ERAD (ER-associated degradation). Furthermore, continuous fluctuation of the ER luminal Ca^{2+} concentration may function as signaling for many ER functions, including protein and lipid synthesis [60]. Therefore, the efficient functioning of the ER is vital for normal cellular functions [9].

The concentration of Ca^{2+} in the extracellular space is between 1 mM and 10 mM, whereas the concentration of Ca^{2+} in the cytosol $[Ca^{2+}]_i$ is in the order of 01 μM thus creating a large inwardly directed electrochemical gradient forcing Ca^{2+} entry across the plasma membrane [67]. Cells have several mechanisms for maintaining a low

$[Ca^{2+}]_i$, which at the same time also ensures that the appropriate transient peak levels of Ca^{2+} are reached during activation. Notable among these are accumulation of the ion into the ER/SR by the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and Na^+ dependent Ca^{2+} uptake (via NCX) [61, 62]. In contrast to the cytosol, where maintenance of low Ca^{2+} concentrations is of vital importance, the ER Ca^{2+} concentration is almost similar to that in the extra cellular space (the ER free Ca^{2+} varies between 200 and 800 μM) [63]. The high ER Ca^{2+} concentration created a second level of Ca^{2+} signaling complexity, as Ca^{2+} ions could enter the cytosol not only from the external space, but also from the intracellular source, ER Calcium efflux from the ER store (generally referred to as Ca^{2+} release) is known to be controlled by several sets of intracellular channels, mainly by the inositol (1,4,5)-triphosphate (IP_3) receptors (IP_3Rs) and ryanodine receptors (RyRs) [63]. Therefore, any defect in the activities of RyRs and IP_3Rs could play a key role in Ca^{2+} dysregulation in the ER as well as in cytosol. As NCX and SERCA are involved in Ca^{2+} sequestration in the ER [13, 14], any defect in their activities could lead to an increase in $[Ca^{2+}]_i$ *in situ* [13, 14]. An increase in $[Ca^{2+}]_i$ causes an increase in calpain activity, which subsequently degrades some intracellular proteins by limited proteolysis [64]. This effect could be more damaging than other reversible calcium dependent processes such as phosphorylation and dephosphorylation [64]. Nicotera *et al* [65] suggested that inhibition of ER Ca^{2+} uptake could manifest a sustained intracellular Ca^{2+} elevation and the demise of cells by activating Ca^{2+}-dependent hydrolytic enzymes including members of the calpain protease family.

Calpain has been shown to be associated with the membrane of ER [42, 47]. It is, therefore, conceivable that calpain is able to cleave ER proteins Indeed several calpain substrates have been identified in the membrane of the ER. In fact the rising evidences indicate that these calcium regulatory signaling proteins or ion channels either cleaved by direct ER calpain or by other cellular calpain during ER stress induced elevated cellular Ca^{2+} level. Therefore, ER calcium and calpain have great role on cellular Ca^{2+} dynamics. In this article we intend to describe how these ER calcium regulatory proteins are cleaved by calpain and its consequence in cellular Ca^{2+} dynamics. The ryanodine receptor (RYR) and inositol triphosphate receptor (IP_3R) are the two Ca^{2+} channels found in the membrane of the ER. The primary physiologic mechanism of RYR channel activation is thought to be increased cytosolic Ca^{2+} (Ca^{2+}-induced Ca^{2+} release). Digestion of cardiac junctional sarcoplasmic reticulum vesicles by m-calpain causes cleavage of the 400 kDa RYR2 isoform into 350 and 315 kDa intermediates followed by generation of a 150 kDa limit protein [66].

The IP_3R is the second Ca^{2+} channel in the ER IP_3R channel opening is stimulated by binding of IP_3, a second messenger generated when phospholipase C converts phosphatidylinositol bisphosphate (PIP2) to IP3 and diacylglycerol. There is also evidence that IP3R Ca^{2+} release is enhanced by increased intracellular Ca^{2+}, similar to RYR Calpain-mediated proteolysis of IP3R1 from the rat cerebellum or striatum results in loss of intact protein (260 kDa) and generation of 200, 130, and 95 kDa (predominant) fragments [67]. This proteolysis of the IP3R resulted in decreased IP_3 binding, suggesting that site-specific cleavage decreases the affinity of the remaining protein species for the IP_3 ligand. A possible calpain-mediated

reduction in ligand binding is consistent with studies of IP_3R in postischemic brain [68]. Recent research also demonstrated that pulmonary smooth muscle ER luminal m-calpain large subunit (80 kDa) cleaved the IP_3R1 luminal (L3) loop and produced a 40 kDa IP_3R1 fragment in a Ca^{2+} dependent manner. This cleavage could lay a key role in Ca^{2+} dysregulation of cell due to inability to release Ca^{2+} from the ER [45].

The sarcoplasmic/ER Ca^{2+}ATPase (SERCA) is responsible for energy-dependent ER Ca^{2+} sequestration Sarcoplasmic/ER Ca^{2+}ATPase has been shown to be susceptible to calpain proteolysis *in vitro*, but only when the ATPase is in its oxidized form Although calpain-mediated degradation of SERCA has been shown after ischemia–reperfusion injury in the heart [69], little data exist for the effects of ischemia on brain SERCA Parsons *et al* [70], has measured SERCA ATPase activity in brain microsomes after decapitation ischemia. Sarcoplasmic/ER Ca^{2+}ATPase function has not yet been examined in the reperfused brain where conditions favor SERCA oxidation. Still, loss of SERCA function because of calpain-mediated proteolysis, like calpain alteration of RYR and IP_3R systems, is a potential mechanism of Ca^{2+} overload in postischemic neurons.

The presence of Na^+/Ca^{2+} exchanger (NCX) in ER and NCX mediated Ca^{2+} uptake in ER have been reported [62, 71]. As Ca^{2+} uptake is involved in Ca^{2+} sequestration in ER of contractile tissues [13, 14], any defect in its activity could lead to an increase in $[Ca^{2+}]_i$ *in situ* [13, 14]. An increase in the mobilization of Ca^{2+} in pulmonary smooth muscle has been suggested to be an important mechanism for pulmonary hypertension and edema [72]. Recently, Samanta *et al* [54] reported that Ca^{2+} dependent dissociation of the associated m-calpain and calpastatin in the ER membrane activates the m-calpain, which consequently cleaves the ER integral transmembrane protein NCX1 (116 kDa) to an 82 kDa fragment. They also suggested that the calcium-dependent proteolysis of NCX1 by m-calpain in the ER plays an important role in intracellular Ca^{2+} overload, which could be important for the manifestation of pulmonary hypertension [13, 14].

5 ER, Calpain and Apoptosis

Accumulating evidences suggest that in addition to mitochondria, the ER serves as an important apoptotic control point. Antiapoptotic BCL-2 and proapoptotic BAX, BAK also localize to the ER Overexpression of BCL-2 was noted to prevent cell death by the passive release of ER Ca^{2+} when thapsigargin was used to block the sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPase reuptake pump [73]. Either over expression of BCL-2 or loss of BAX, BAK leads to reduced resting ER Ca^{2+} concentrations and a secondary decrease in Ca^{2+} uptake by mitochondria [74, 75]. Selective reconstitutions have enabled the classification of death signals based on their dependence on an ER Ca^{2+} gate-way and/or a mitochondrial gateway to apoptosis [75]. Signals reliant upon the ER gateway include the Ca^{2+}-dependent lipid second messengers such as, C_2-ceramide and arachidonic acid as well as pathologic oxidative stress [76].

In the lumen of the ER, Ca^{2+} is stored, and disturbance of the Ca^{2+} homeostasis initiates ER stress, which leads to apoptosis [77]. The activation of the unfolded protein response (UPR) induces an adaptative response in which the cell attempts to overcome the accumulation of misfolded proteins and ER stress [77]. In certain cases, however, the activation of the UPR may be insufficient to overcome ER stress and the continued accumulation of misfolded proteins in the ER may have toxic effects, ultimately leading to cell death. Apoptosis is an important mechanism to induce cell death in the context of ER stress, but the mechanisms that regulate ER stress induced apoptosis are still not clear. Caspases are cysteine proteases that exist within the cell as inactive pro-forms or zymogens and are cleaved to form active enzymes following the induction of apoptosis. Caspase-12 is important in the context of ER stress induced apoptosis [78]. Caspase 12 is activated by calpains [78] during ER stress induced increase of $[Ca^{2+}]_i$ under a variety of pathological conditions (Fig. 2) [77]. Recent research by Samanta *et al* [54] demonstrated that in elevated level of calcium, ER membranal and luminal m-calpain become activated, which could activate the ER protein procaspase 12 (Fig. 2) After its activation at the ER, caspase-12 cleaves procaspase-9, leading to caspase-9-dependent activation of caspase-3 [78] (Fig. 2). ER stress induced processing of procaspase-9 can occur in the absence of cytochrome *C* release, which argues that caspase-12 triggers directly caspase-9 activation and apoptosis, independently from the mitochondrial cytochrome *C*/Apaf-1 pathway [78]. The role of caspase-12 in human cells is, however, not clear, as the gene contains several mutations impairing its function [78]. Other caspases, for example, caspase-4 [78] may also be involved in ER stress induced apoptosis in human cells. Mitochondrial involvement (or cross talk between the ER and mitochondria) in ER stress-induced cell death has been shown by the release of cytochrome *C* from mitochondria after induction of ER stress [77]. Members of all Bcl-2 classes are localized on the intracellular membrane of ER and regulate ER Ca^{2+} homeostasis, probably by influencing membrane permeability Ca^{2+} release may induce apoptosis by influencing the mitochondrial permeability transition pores (PTP) or by direct activation of caspase-12 [77].

6 ER Calpain in Pathophysiology

ER has important roles in the regulation of cytosolic Ca^{2+} dynamics by taking it up when cytosolic Ca^{2+} levels are high and releasing it when cytosolic Ca^{2+} levels are low [7]. In some pathophysiological conditions, this $[Ca^{2+}]_i$ homeostasis may be dysregulated by ER stress, which activates calpain that cleaves the ER resident Ca^{2+} regulatory proteins or other signaling proteins. Thus, calpain mediated cleavage of these proteins in the ER is the main manifestation of the cellular dysfunctions that may lead to cell death (Fig. 2). Under some pathophysiological conditions, disturbance of ER Ca^{2+} homeostasis leads to the intracellular Ca^{2+} overload, which could be important for the manifestation of pulmonary hypertension [79, 80]. Recently, Samanta *et al* [54] reported that the activated m-calpain cleaves the ER integral

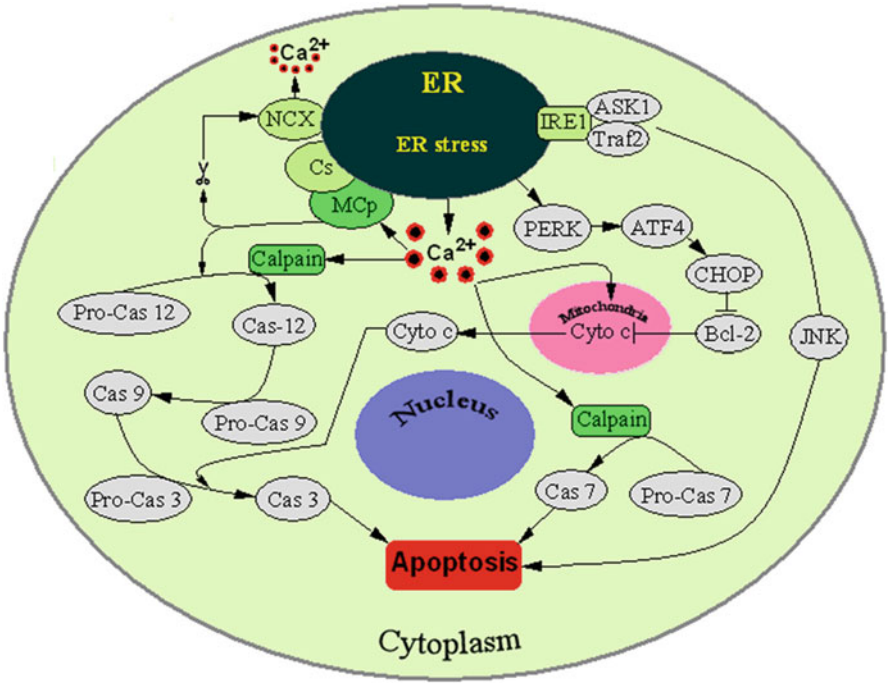

Fig. 2 Mechanisms of induction of apoptosis by ER stress. The direct activation of caspases-12 by Ca^{2+} dependent calpain activates the caspase-9/caspase-3 cascade. Overload of mitochondria of Ca^{2+} from the ER leads to the release of proapoptotic factors, such as Cyt *C*, from mitochondria. Activation of CHOP (C/EBP homologous protein) by PERK/eIF2a/ATF4 (protein kinase RNA-like ER kinase/eukaryotic initiation factor-2/activating transcription factor 4) leads to the upregulation of pro-apoptotic and down regulation of antiapoptotic Bcl-2 family members IRE1/Traf2/ASK2 (inositol requiring protein-1/tumour necrosis factor receptor-associated factor 2/ASK2) activates JNK (c-Jun N-terminal Kinase) signaling to trigger apoptotic pathways ER stress-mediated increased Ca^{2+} may also activate the ER associated MCp, which then activates the pro-caspase 12 and induces caspase 12 mediated apoptosis and this activated MCp may also cleave the ER Ca^{2+} uptake protein NCX, which is the main cause of secondary Ca^{2+} overload in the cytosol and cellular dysfunction

trans-membrane protein NCX1 (116 kDa) to an 82 kDa fragment. It has also been suggested that the calcium-dependent proteolysis of NCX1 by m-calpain in the ER plays an important role in intracellular Ca^{2+} overload, which could be important for the manifestation of pulmonary hypertension [12, 13].

The spatial and temporal activity of calpain suggests a possible causal role for calpain in postischemic neurodegeneration [68, 81]. The pathology of ischemic neuronal injury involves alterations in the function of multiple cellular components, including the synapse, plasma membrane, ER, mitochondria, lysosomes and nucleus. All these neuronal regions or organelles contain calpain substrates, and, therefore, a systematic consideration of the subcellular localization of pathologic calpain activity is one method by which one can understand the mechanism of calpain-mediated

cell death. Calpain substrates also exist across a wide range of functional categories. Calpain cleavage of this set of targets leads directly to cellular breakdown that is a hallmark of postischemic neuronal death.

Calpain-mediated proteolysis of different Ca^{2+} regulating proteins and ion channels such as RyR, IP_3R, and SERCA in the ER are thought to be a potential mechanism of cytosolic Ca^{2+} overload [68]. Calpain-mediated cleavage of RYR2 renders the channel unable to close in the presence of elevated cytosolic Ca^{2+} levels RYR2 channels in a 'locked open' state are a potential mechanism for permanent ER Ca^{2+} depletion and loss of the ER's ability to buffer elevations in cytosolic Ca^{2+}. Postischemic administration of the RYR channel antagonist, dantrolene has been reported to be neuroprotective both *in vitro* [82] and *in vivo* [83], suggesting that increased Ca^{2+} efflux via RYR could contribute to sustained or secondary postischemic calpain activation and eventual neuronal death.

Recent research by Kopil *et al* [84] demonstrated that expression of calpain-cleaved $InsP_3R1$ impairs neuronal ER Ca^{2+} buffering, leading to increased sensitivity to excitotoxic stimuli and neurodegeneration in primary neuron cultures. As an early part of the molecular injury cascade [85], calpain proteolysis of $InsP_3R1$ could contribute a feed forward pathway to accelerate neuronal death through $[Ca^{2+}]_i$ overload. Therefore, inhibiting Ca^{2+} release through calpain-cleaved $InsP_3R1$ is a potentially important therapeutic strategy for intervention in disorders associated with calpain activity and disruption of Ca^{2+} homeostasis Samanta *et al* [45] reported that the treatment of the ER isolated from bovine pulmonary artery smooth muscle with the Ca^{2+} ionophore, A23187 causes activation of ER luminal m-calpain, which subsequently cleaves IP_3R1. They also suggested that the calcium-dependent proteolysis of NCX1 by m-calpain in the ER plays an important role in intracellular Ca^{2+} overload [54]. This group suggested that these are the main cause of Ca^{2+} accumulation in the cytosol as well as in the ER lumen and that could play important role in pulmonary hypertension ER stress has been implicated in the etiology of multiple neurodegenerative disorders, including Huntington's disease and Alzheimer's disease [86].

7 Conclusion and Future Perspective

The present review emphasizes on the ER calpain system, an emerging area of calpain research. Recent research demonstrated that ER contains MCp, LCp and calpain endogenous inhibitor, calpastatin [42, 45, 47]. These calpains are regulated by calpastatin and or ERp group of protein, ERp57 [42, 45, 47]. Current research established that the association between calpains and its endogenous inhibitor, calpastatin in several cellular and subcellular organelles [41–43, 47]. Ideally, this interaction of the protease and its physiological inhibitor is the main regulatory mechanism of several pathophysiological interventions. Therefore, crystallographic structure of the associated calpains and calpastatin would be beneficial for better understanding the appropriate regulation of the calpain activity by calpastatin.

Proper folding of proteins is aided by multiple chaperones functioning in the endoplasmic reticulum (ER). Misfolded or unfolded proteins and stimuli that disrupt ER function initiate the unfolded protein response (UPR), a stress response pathway by a transcriptional program, thereby ensuring upregulation of key proteins necessary to restore proper "protein homeostasis" [87]. In the extreme, if proper protein folding is not achieved, unfolded proteins can trigger apoptosis ER stress has been implicated in the etiology of multiple neurodegenerative disorders, including Huntington's disease and Alzheimer's disease [86]. Therefore, ER calpain is pathophysiologically important.

SR/ER Ca^{2+} ATPase has been shown to be susceptible to calpain proteolysis *in vitro*, but only when the ATPase is in its oxidized form Calpain-mediated degradation of SERCA has been repeatedly shown after ischemia-reperfusion injury in the heart [75]. Loss of SERCA function because of calpain-mediated proteolysis, like calpain alteration of RYR and IP_3R systems, is a potential mechanism of Ca^{2+} overload in postischemic neurons. An important question that could be raised about the mechanism(s) by which calpain activation occurs in an oxidant environment in cells as well as the direct involvement of the ER MCp or LCp in these neurological disorders. Hence, functional regulation of ER calpain activity in several pathophysiological conditions is an important aspect of future research.

Regulation of calpain activity stands as an important issue in developing therapeutic reagents for heart failure [88, 89]. In this respect, it should be noted that some pathological states may result from insufficient calpain activity. Genetic defects in tissue-specific calpains cause various diseases, which may be improved by activators or stabilizers for these calpains. Similarly, determination of the involvement of several subcellular organelles specific calpain like ER is also an important filed of future research. One of the most urgent needs in this research is the ability to detect real-time organelles specific calpain activity *in vivo* at a high resolution for both physiological and pathophysiological conditions. As calpain research enters this new era, both basic science and translational biomedical studies are well positioned to launch comprehensive calpain studies. Finally, with the completion of the study with ER calpain system, one expects involvement and specific regulation of ER calpain in several pathophysiological interventions to be studied at a faster pace.

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Mitochondrial Calpains: Who, What, Where, When and Why?

James W. Geddes

Abstract Evidence accumulated over the past two decades has clearly demonstrated that one or more calpains are localized to mitochondria. This evidence includes mitochondrial calpain activity, immunoreactivity, purification, and N-terminal sequencing. Supporting data is greatest for CAPN1 and CAPN10, although the support for mitochondrial CAPN2 is also compelling. The mitochondrial localization of calpains may protect them from CAST inhibition, and also expose the proteases to Ca^{2+} transients during mitochondrial Ca^{2+} accumulation and/or opening of the mitochondrial permeability transition pore. One suggestion is that calpain activation may be required for mitochondrial permeability transition, with much stronger evidence demonstrating calpain activation in the aftermath of permeability transition pore opening. Several putative substrates of mitochondrial calpains have been identified, with the greatest attention paid to apoptosis inducing factor. Although much data are consistent with the involvement of mitochondrial CAPN1 activation in the processing and release of apoptosis inducing factor, there are also contrasting data. All mitochondrial calpains identified to date are also present in the cytosol, making selective mitochondrial inhibition or knockdown difficult and complicating analysis of mitochondrial calpains. Calpains are clearly present in mitochondria, much work remains to identify their substrates and physiological and pathological roles.

Keywords Apoptosis • Necrosis • Calcium • Cell Death • Apoptosis inducing factor

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1 Introduction

Calpains are a family of Ca^{2+}-activated cysteine proteases (Clan CA, family C2, EC 3.4.22.52-54) which cleave their substrate proteins at one or more sites to modulate protein activity [1, 2]. The calpain family, including the small subunits and endogenous inhibitor calpastatin, are encoded by 17 genes in mammals (Table 1). The best characterized calpains are the ubiquitous, classical calpains CAPN1[μCL] and CAPN2[mCL]. This nomenclature is based on the recently proposed unified nomenclature for calpains [2]. CAPN1[μCL] and CAPN2[mCL] were identified based on their different calcium requirements, with purified CAPN1[μCL] requiring μm Ca^{2+} for activation and CAPN2[mCL] being activated by mM Ca^{2+} [3, 4]. They are heterodimers consisting of a unique 80 kDa large subunit (CAPN1 and 2) and a common 28 kDa small subunit (CAPNS1). Several additional members of the calpain family are also ubiquitously expressed (CAPNs 5, 7,10,13-15) while other calpains are tissue-specific (CAPNs 3,6,8,9,11,12). Calpains are also subdivided based on primary sequence as classical calpains (CAPNs 1-3, 8,9,11-14) and non-classical calpains (CAPNs 5-7,10,15,16) (for recent review see [2]). Classical calpains

Table 1 The calpain family (modified from [2])

Product name	Previous name	Gene	Distribution	Classification	Notes/other names
CAPN1	Calpain 1	Capn1	Ubiquitous	Classical	
CAPN2	Calpain 2	Capn2	Ubiquitous[a]	Classical	
	μ-Calpain		Ubiquitous	Classical	CAPN1/CAPNS1
CAPN1[μCL]	m-Calpain		Ubiquitous	Classical	CAPN2/CAPNS1
CAPN3	Calpain 3	Capn3	Skeletal muscle	Non-classical	P94
CAPN5	Calpain 5	Capn5	Ubiquitous	Non-classical	hTra-3
CAPN6	Calpain 6	Capn6	Placenta, embryonic muscle[b]	Non-classical	
CAPN7	Calpain 7	Capn7	Ubiquitous	Non-classical	PalBH
CAPN8	Calpain 8	Capn8	Gastrointestinal	Classical	nCL-2
CAPN9	Calpain 9	Capn9	Gastrointestinal	Classical	nCL-4
CAPN10	Calpain 10	Capn10	Ubiquitous	Non-classical	
CAPN11	Calpain 11	Capn11	Testis	Classical	μ/mCL in avians
CAPN12	Calpain 12	Capn12	Hair Follicles	Classical	
CAPN13	Calpain 13	Capn13	Ubiquitous	Classical	
CAPN14	Calpain 14	Capn14	Ubiquitous	Classical	
CAPN15	Calpain 15	Capn15	Ubiquitous	Non-classical	SOLH
CAPN16	Calpain 16	Capn 16	Ubiquitous	Non-classical	Demi-Calpain, C6orf103
CAPNS1	Small subunit 1	CapnS1	Ubiquitous	Classical	Calpain 4
CAPNS2	Small subunit 2	CapnS2	Ubiquitous		
CAST	Calpastatin	CAST	Ubiquitous	Inhibitor	

[a]CAPN2 is present in all cells with the exception of erythrocytes
[b]CAPN6 lacks Cys at the active site and is non-proteolytic

contain a penta EF-hand domain as well as a CS-like domain. Non-classical calpains lack either or both of these domains [2]. Calpains are inhibited by a single endogenous inhibitor, CAST (calpastatin) [5].

Since the discovery of calpain activity by Guroff and colleagues [6], a tremendous amount of information has been obtained regarding the properties of calpains, their substrates, tissue and cellular localization, physiologic functions, and potential role in various diseases (for review see [7, 8]). However, significant questions remain. In most cells and tissues, CAST activity exceeds the combined activities of CAPN1[μCL] and CAPN2[mCL] [9]. Thus, it is unclear how calpains become activated. The association of CAST with the classical calpains is promoted at lower Ca^{2+} levels than are required for calpain activation, and both the calpains and their inhibitor are localized to the cytosol [10]. Calpain localization in an organelle would protect it from CAST-mediated inhibition. Below we discuss the considerable accumulated evidence demonstrating the mitochondrial localization of three members of the calpain family: CAPN1[μCL], CAPN2[mCL], and CAPN10.

2 Evidence for Mitochondrial Calpains

The earliest suggestion of a mitochondrial calpain was based on Ca^{2+}-activated protease activity in rat liver mitochondria[11]. Beer and colleagues identified two peaks of protease activity, one which was half-maximally activated by 25 μM Ca^{2+} and the other requiring 750 μM Ca^{2+}. Together, this mitochondrial activity accounted for approximately 10 % of the Ca^{2+}-dependent protease activity of rat liver homogenates [11]. The 10 % estimate of mitochondrial calpain is similar to the findings of an ultrastructural study of skeletal muscle calpain [12]. Beer et al. also noted that a previously described neutral protease identified in rat liver mitochondria had many similarities including inhibition by EGTA and leupeptin [13]. Tavaras and colleagues identified three Ca^{2+}-activated proteolytic activities in the matrix of rat liver mitochondria, two of which were also detected in the intermembrane space [14]. In rat retinas, the cleavage of mitochondrial aspartate aminotransferase under hypoxic and ischemic conditions was attributed to calpain-mediated proteolysis in mitochondria [15].

In rat brain, subcellular localization found the highest activity of CAPN2[mCL]-like activity was detected in the soluble cytoplasmic fraction, while the CAPN1[μCL] activity was greatest in the crude mitochondrial fraction [16–18]. Vigorous homogenization was necessary for isolation of CAPN1[μCL], suggesting an association with subcellular structures [9].

Additional evidence for a mitochondrial calpain includes the observation that mitochondrial proteins including AIF and mitochondrial Bax are calpain substrates [19, 20]. Mitochondrial calpain-like protease activity is implicated in the initiation of mitochondrial permeability transition and cell necrosis in an experimental model of cholestasis [21]. Cysteine proteases are released from mitochondria, along with cytochrome C, following incubation of the unicellular eukaryote Leishmania major with recombinant human Bax, suggesting that the relationship between

mitochondria cysteine proteases and cell death pathways may be of ancient evolution origin and be highly conserved [22]. Further evidence of a mitochondrial calpain was the observation that cyclosporine A, which binds to cyclophilin D to inhibit opening of the mitochondrial permeability transition pore, can prevent calpain activation in some situations [23–25]. There is also considerable evidence that mitochondrial dysfunction observed in necrotic/oncotic cell death is mediated by a mitochondrial calpain of unknown identity [26].

3 Identification of Mitochondrial Calpains

Based on the above, several groups sought to identify this unknown mitochondrial calpain or calpains. Using antibodies against various domains of CAPN1 and CAPNS1, we found that CAPN1[μCL] was enriched in mitochondria isolated from rat cortex and SH-SY5Y neuroblastoma cells and localized to the mitochondrial intermembrane space [27, 28]. The N-terminus of CAPN1 contains an extra 12-14 amino acids that are not present in CAPN2 [29]. This is similar to the amphipathic N-terminal sequence of other IMS proteins (i.e. AIF, endonuclease G, Smac/Diablo, HtrA2/Omi) that facilitates their interaction with mitochondrial import machinery [30]. Mitochondrial import studies and localization of fusion proteins confirmed that the N-terminus of CAPN1 represents a mitochondrial targeting sequence [28].

Kar and colleagues found both components of CAPN1[μCL], the CAPN1 large subunit and CAPNS1 small subunit, to be present in mitochondria isolated from bovine pulmonary artery smooth muscle, along with CAST [31]. Identity of the proteins was confirmed following purification and amino-terminal amino acid sequence analysis. Both CAPN subunits were localized to the inner mitochondrial membrane [32]. Kar et al. nicely summarized the mitochondrial calpain system in a previous review [33].

In a mitochondrial fraction prepared from swine liver, Ozaki and colleagues partially purified a mitochondrial calpain using column chromatography [34]. This calpain had biochemical properties similar to cytosolic CAPN1[μCL] and CAPN2[mCL], but differed in pH optimum and motility on zymography [34]. This protein was subsequently identified as mitochondrial CAPN1[μCL] located in the mitochondrial intermembrane space, and associated with the protein-disulfide isomerase ERp57 [35]. CAST was not detected in the swine liver mitochondria.

Based on the previous report from Beer [11] identifying at least two types of calpain activity in mitochondria, Ozaki and colleagues sought to further characterize calpains present in mitochondria and found that CAPN2[mCL], was also present in rat liver mitochondria, where it was associated with Grp75 in the intermembrane space [36]. CAPN2[mCL], was also found bound to the outer mitochondrial membrane, where it was released following incubation with Ca^{2+} [37]

Arrington, Schnellman and colleagues identified an atypical calpain, CAPN10, as a mitochondrial calpain in rat and rabbit kidney mitochondria [38]. CAPN10 was localized to several submitochondrial fractions, including the outer membrane,

intermembrane space, inner membrane, and matrix. As with CAPN1, the N-terminus of CAPN10 contains a mitochondrial targeting sequence. Overexpression of CAPN10 resulted in mitochondrial swelling and autophagy, and substrates included components of Complex I of the electron transport chain. Three splice variants of CAPN10 were identified in the kidney of various mammalian species [39], with renal expression decreasing with advancing age [40]. A small peptide, CYGAK, was identified as a specific calpain 10 inhibitor [41].

CAPN1[μCL] and CAPN2[mCL] have also been localized to other subcellular organelles including Golgi apparatus, endoplasmic reticulum, and nuclear fractions [42–45].

4 Mitochondrial Calpain Substrates

Although a consensus has emerged that calpains are present in mitochondria (Table 2), identification of the substrates of mitochondrial calpains has proven elusive. The mitochondrial substrate which has attracted the most attention is apoptosis inducing factor (AIF). Mature 62 kDa AIF is anchored to the mitochondrial inner membrane and projects into the intermembrane space [46]. It has an NADH-oxidase domain and serves physiological roles related to redox regulation and mitochondrial bioenergetics [47, 48]. An unidentified protease cleaves AIF to the 57 kDa form (truncated AIF or tIAF), which is released into the cytoplasm and then translocates to the nucleus where it induces DNA fragmentation, chromatin condensation, and caspase-independent cell death [46, 47, 49, 50]. Similar to AIF, the release of endonuclease G requires partial proteolysis by an unidentified protease [51]. Unresolved is whether the protease responsible for AIF cleavage is intramitochondrial or is a cytosolic protease that gains access to the mitochondrial IMS following permeabilization of the mitochondrial outer membrane [20, 46].

Data supporting the involvement of a mitochondrial calpain in AIF cleavage and release has been provided by several investigators. Polster and colleagues demonstrated that AIF is CAPN1[μCL] substrate, and that release of tAIF from isolated rat liver and brain mitochondria is facilitated by outer mitochondrial membrane permeabilization and addition of active calpain, and inhibited by calpeptin [20]. Moreover, Ca^{2+}-induced release from rat liver mitochondria was inhibited by cyclosporine A which impairs opening of the permeability transition pore. A sustained

Table 2 Mitochondrial calpains identified to date

Calpain	Location	References
CAPN1[μCL]	Intermembrane space, inner membrane	[11, 14, 27, 28, 31, 32, 34, 35]
CAPN2[mCL]	Intermembrane space, outer membrane	[11, 14, 36, 37]
CAPN10	Outer membrane, intermembrane space, inner membrane, matrix	[14, 38]
CAST	Inner mitochondrial membrane	[31, 32]

elevation in intracellular Ca^{2+} was required for activation of mitochondrial calpain and AIF release from U1810 cells, and AIF release was prevented by calpain inhibition or Ca^{2+} chelation, although the calpain isoform involved was not investigated [52]. Knockdown of CAPN1 using small interfering RNA, or overexpression of CAST, abolished ischemia-induced translocation of AIF from mitochondria to the nucleus in a rat model of transient global ischemia and in neuronal cultures challenged with hypoxia-hypoglycemia [53] or NMDA [54]. In cisplatin-induced death of human lung adenocarcinoma cells, calpain inhibitors attenuated both AIF translocation and caspase 9/3 activation, implicating calpains in both caspase-dependent and –independent cell death [55]. Calpains are also implicated in mitochondrial AIF release following transient focal ischemia [56]. Although not involved in AIF processing to tAIF, CAPN2[mCL] is implicated in the release of tAIF via cleavage of the voltage dependent anion channel on the outer mitochondrial membrane [36, 37].

While there is substantial support for the involvement of calpains in AIF processing and release, there are also contrasting opinions. While broad-spectrum calpain inhibitors inhibit AIF release from rat liver mitochondria, more specific calpain inhibitors are less effective [57, 58]. Moreover, Ca^{2+}-induced activation of CAPN1[μCL] in mitochondria isolated from SH-SY5Y cells is not accompanied by AIF truncation and release [57]. Similarly, calpain activation is not required for AIF translocation in PARP-1-dependent cell death [59]. Whether calpains or other cysteine proteases are responsible for AIF processing and release is not fully resolved

Another mitochondrial substrate implicated in cell death is Bid. Bid is cleaved by several proteases including calpains to a truncated form, tBid, which then interacts with Bax to cause translocation to the outer mitochondrial membrane, resulting in outer membrane permeabilization and release of proteins from the intermembrane space [60, 61]. Shulga and Pastorino demonstrated that tBid can activate mitochondrial CAPN1, and that this activation is facilitated by acyl coenzyme A-binding protein [62].

Additional mitochondrial calpain substrates include the Na^+/K^+ exchanger as a substrate of CAPN1 [63], and two components of complex I of the electron transport chain, NDUFV2, and ND6, as substrates of CAPN10 [38]. As indicated above, mitochondrial aspartate aminotransferase is also a putative mitochondrial calpain substrate [15]. Opa 1 has not been demonstrated to be cleaved by calpains, but protecting Opa 1 from calpain activity protects neurons from excitotoxic death [64].

5 Role of Calpains in Cell Death

Unlike the well-defined role of caspases in caspase-dependent apoptosis [65], the role of calpains in cell death pathways are not well understood. Calpains can play an essential [73] or augmentative role in caspase-dependent apoptosis [66], and may also play an essential role in necrotic/oncotic death [26, 67]. It is therefore of interest, that levels of CAPN1 and caspase 3 are reciprocally regulated during brain

development [68], with caspase 3 being elevated in the embryonic and early postnatal brain when caspase-dependent cell death predominates and CAPN1 being elevated in the late postnatal and adult brain, when necrotic death morphology is prominent following insult.

The mechanism by which calpain activation results in cell death is unclear. One possibility is that proteolysis of cytoskeletal substrates is responsible for the cell death, as disruption of the cytoskeleton is an early event in neurodegenerative cascades [69]. Other potentially lethal calpain substrates include the plasma membrane Na^+/Ca^{2+} exchanger [70]; the cdk5 activator p35, which when cleaved by calpain to p25 is resistant to degradation and induces cell death [71, 72]; and the bcl-2 related proteins Bid and Bax [19, 73, 74]. Opa1, the mitochondrial inner membrane GTPase, is critical in excitotoxic neuron death and is downstream of calpain. Either calpain inhibition or Opa1 overexpression can protect neurons [64], suggesting that calpain cleavage of Opa1 on the inner mitochondrial membrane plays a critical role in excitotoxic neuron death. Additionally, calpain may cleave apoptosis inducing factor (AIF), anchored to the inner mitochondrial membrane, to induce caspase-independent cell death [20, 58]. This is discussed in greater detail below.

6 The Mitochondrial Permeability Transition Pore and Calpain Activation

When mitochondria become overloaded with Ca^{2+}, particularly under conditions of oxidative stress and low ATP levels (such as following TBI), they undergo permeability transition via formation of a nonselective pore that allows Ca^{2+} and other solutes of 1,500 daltons or smaller to pass through the usually impermeable inner mitochondrial membrane with a resultant rupture of the outer mitochondrial membrane caused by osmotic swelling (for review see [75]). Opening of the mPTP is associated with necrotic, caspase-independent, cell death. This necrotic death is minimized in mice that do not express cyclophilin D, which gates the opening of the mPTP in response to elevated Ca^{2+}, while apoptotic death is unaffected in the cyclophilin D null mice.

Evidence supporting the hypothesis that calpain(s) are activated by opening of the mPTP includes the observation that cyclosporine A, which inhibits cyclophilin D-mediated opening of the mPTP, prevents calpain activation in neurons following transient hypoglycemia [25] in hepatocytes following microcystin [24] and limits spectrin and neurofilament proteolysis following TBI [23]. Cyclosporine A provides significant protection against neurodegeneration following focal TBI [76, 77]. In addition, in a study from Farkas et al. [78] calpain-cleaved α-spectrin breakdown products surrounded swollen mitochondria following diffuse traumatic brain injury. [79–82]. In oligodendrocytes, excessive mitochondrial Ca^{2+} accumulation results can result in apoptosis which is inhibited by blocking mitochondrial Ca^{2+} influx or calpain inhibitors [83].

7 Conclusions

In summary, evidence has accumulated over the past two decades to clearly demonstrate that one or more calpains are localized to mitochondria. This evidence includes mitochondrial calpain activity, immunoreactivity, purification, and N-terminal sequencing. Supporting data is greatest for CAPN1 and CAPN10, although the support for mitochondrial CAPN2 is also compelling. The mitochondrial localization of calpains may protect them from CAST inhibition, and also expose the proteases to Ca^{2+} transients during mitochondrial Ca^{2+} accumulation and/or opening of the mitochondrial permeability transition pore. One suggestion is that calpain activation may be required for mitochondrial permeability transition, with much stronger evidence demonstrating calpain activation in the aftermath of permeability transition pore opening. Several putative substrates of mitochondrial calpains have been identified, with the greatest attention paid to AIF. Although much data are consistent with the involvement of mitochondrial CAPN1 activation in AIF processing and release, there are also contrasting data. All mitochondrial calpains identified to date are also present in the cytosol, making selective mitochondrial inhibition or knockdown difficult and complicating analysis of mitochondrial calpains. Calpains are clearly present in mitochondria, yet much work remains to identify their substrates and physiological and pathological roles.

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Role of Calpains in Calmodulin Regulated Systems

Sreejit Parameswaran, Sujeet Kumar, and Rajendra K. Sharma

Abstract Dysregulation of proteolytic enzymes may disrupt normal biological processes in myocardium can lead to various cardiac conditions. Substantial evidence supports the involvement of matrix metalloproteinase, cystine and serine protease families in this process. Calpain is an intracellular Ca^{2+}-activated protease. Deregulation of calpain caused by a disruption of calcium homeostasis during cardiac pathologies such as atrial fibrillation, heart failure, hypertrophy, or ischemia reperfusion, and thus the myocardial damage. Calpain-calcineurin signalling is pivotal in cardiac conditions especially ischemia, since the signalling produces a cascading effect on the outcome of ischemia. The cleavage of phosphodiesterase1 by calpain is crucial for the regulation of cyclic nucleotides especially cAMP and cGMP. Turnover of cAMP and cGMP in cardiac tissue determines how the cells respond and survive to ischemic insult. Among the known calpain inhibitors, the most specific and potent inhibitor is calpastatin, which belongs to the calpain family. Further research on calpain structures will help in determining the conditions required for activation and the ability of calpain specifically proteolyse even untagged substrates. Though many interesting reviews have covered on the entire calpain system, the current manuscript focuses on the research carried out on calpains in relation to cardiac system by describing their interaction with 2 important cardiac specific proteins—calcineurin and phosphodiesterase 1.

Keywords Calpain • Cardiac protease • Calcineurin • Phosphodiesterase • Calpain inhibitors

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1 Introduction

Cardiovascular diseases are the leading cause of deaths worldwide [1]. Although various factors have been identified as possible causes of different cardiac diseases such as heart failure and ischemic heart disease, there is a real need to elucidate their role for the better understanding of the cardiac disease pathology and formulation of strategies for developing newer therapeutic interventions. In view of the intimate involvement of different types of proteases in maintaining cellular structure, the role of proteases in various cardiac diseases has become the focus of recent research [2]. Proteases are present in the cytosol as well as are localized in a number of subcellular organelles in the cell. These are known to use extracellular matrix, cytoskeletal, sarcolemmal, sarcoplasmic reticular, mitochondrial and myofibrillar proteins as substrates. The most common proteolytic enzyme with regards to heart disease is cathepsin [3, 4]. Other proteolytic enzymes are matrix metalloproteinases, calpains, and caspases [5, 6]. In addition to modifying the extracellular matrix, both matrix metalloproteinases and cathepsins have been shown to affect the activities of subcellular organelles in cardiomyocytes. The activation of calpains and caspases has been identified to induce subcellular remodeling in failing hearts. Proteolytic activities associated with different proteins including caspases, calpains, and the ubiquitin-proteasome system has been shown to be involved in cardiomyocyte apoptosis.

2 Calpains

Calpains are intracellular Ca^{2+}-activated proteases which act as an important mediator of the actions of Ca^{2+}. Cleavage by calpains are critical in a variety of Ca^{2+}-regulated cellular processes such as muscle contraction, neuronal excitability, secretion, signal transduction, cell proliferation, differentiation, cell cycle progression, and apoptosis. Deregulation of calpains caused by a disruption of Ca^{2+} homeostasis during cardiac pathologies such as atrial fibrillation, heart failure, hypertrophy, or ischemia reperfusion, is critically involved in the myocardial damage [7]. Calpain-mediated proteolysis is mediated by a family of Ca^{2+}-dependent, non-lysosomal cysteine proteases that are expressed ubiquitously within all cells as inactive proenzymes [8, 9]. The calpain family (EC 3.4.22.17; Clan CA, family C02) consists of at least 16 proteins, all of which are coded for by an independent gene [10, 11]. The two most well studied forms of calpains are μ-calpain (calpain-1) and m-calpain (calpain-2), named for their responsiveness to either micromolar or millimolar concentrations of Ca^{2+}, respectively [12, 13]. Both of these calpains are found in modest amounts within the myocardium, where they have been linked to the degradation of myofibrillar proteins such as the troponins, tropomyosin, titin, desmin, fodrin, filamin, C-protein, nebulin, gelsolin, and vinculin in a variety of cell types, *in vitro* [14].

The calpain system consist of three molecules: two Ca^{2+}-dependent proteases, calpain-1 and calpain-2, and a third polypeptide, calpastatin, whose only known function is to inhibit the first two calpains [15]. Both the calpain-1 and 2 are heterodimers containing an identical 28-kDa subunit and an 80-kDa subunit that shares

55–65 % sequence homology among them. The crystallographic structure of calpain-2 reveals six "domains" in the 80-kDa subunit: (1) a 19-amino acid NH_2-terminal sequence; (2) and (3) two domains that constitute the active site, IIa and IIb; (4) domain III; (5) an 18-amino acid extended sequence linking domain III to domain IV; and (6) domain IV, which resembles the penta EF-hand family of polypeptides [13]. The single calpastatin gene can produce eight or more calpastatin polypeptides (~17 to ~85 kDa) using different promoters and alternative splicing events. The physiological significance of these different forms of calpastatin is unclear, although all bind to three different places on calpain and the binding to at least two of the sites is Ca^{2+}-dependent [14]. Ca^{2+} binding induces a conformational change that leads to assembling of the activated enzyme [13].

The crystallographic structure of calpain-2 shows that calpain-2 at least, and probably calpain-1 also, is catalytically inactive in the absence of Ca^{2+}, since, the three residues constituting the calpain catalytic triad, Cys, His, and Asn, are not sufficiently close in the Ca^{2+} free state to assemble a functional active site [13]. Therefore, calpains cannot be considered as proenzymes in the sense that their active site is sterically blocked by a propeptide. "Knock-out mouse" models has shown that disruption in expression of the small subunit common to both calpain-1 and 2 is embryonically lethal, but that disrupting expression of calpain-1 alone has minimal consequences on survival. It can be assumed that calpain-2 recompensates for the absence of calpain-1. It has been also suggested that calpain-1 and 2 can cleave the same polypeptide substrates, suggesting very similar if not identical subsite specificities of the two calpains [14]. Calpains-1 and 2 require different concentration of Ca^{2+} for their activity [16]. For half maximal activation, calpain-2 requires millimolar concentration of Ca^{2+}, whereas calpain-1 requires micromolar concentration of Ca^{2+} [15]. Recently, it has been reported that calpain-1 activates calpain-2 and the concentration required for its half maximal activation is reduced to micromolar levels [17].

In addition, 12 mammalian mRNAs encoding polypeptides homologous to domains IIa and IIb of the 80-kDa subunit of calpain-1 and 2 had been identified through cDNA cloning. Calpain-like mRNAs have been identified in other organisms. In some instances, these calpain homologs are expressed in specific tissues, and in several instances, the calpain homolog does not have all three residues necessary to form a catalytic triad [14, 15]. Calpains known as "Typical" EF-hand calpains which includes calpains 3, 8, 9, 11, 12 and 13 have a structure similar to the classical calpains [18]. However, these calpains do not seem to form functional heterodimers with the regulatory subunit and only calpain-12 is ubiquitously distributed [19]. Among these, calpain-3 is specific to skeletal muscle, where it has been suggested to assist in the disassembly of the multiprotein complexes which make up the sarcomere, prior to the ubiquitin-proteasome system mediated degradation of the individual sarcomeric proteins [14]. Besides these, a group of "Atypical" calpains have been observed and includes calpains 5, 6, 7, 10, 13 and 15. These are characterized by the absence of the penta-EF-hand domain. The calpains of this subfamily can be further classified according to the protein organization beyond the proteolytic domain II and its homology with the *Caenorhabditis elegans* TRA-3 (TRAnsformer: XX animals transformed into males) calpain [19].

The physiological functions of the calpain system remains relatively vague, despite a great deal of work showing involvement of calpains in cellular functions as diverse as cytoskeletal/plasma membrane attachments, cell motility, signal transduction pathways including activation of some signaling molecules and assembly of focal adhesions, aspects of the cell cycle and regulation of gene expression, and some but not all apoptotic pathways, in addition to roles in other processes such as long-term potentiation [14]. It seems likely that the calpains are capable of cleaving a large number of different "physiological substrates". However, not all calpain-susceptible molecules may be cleaved in a given cellular event; the degradation of molecules may depend on the signals that the cell has received, the location of the calpains (and possibly calpastatin), and possibly other factors. Deregulated calpain activity following loss of Ca^{2+} homeostasis, often results in tissue damage especially in response to events such as myocardial infarcts, stroke, and brain trauma [14, 15]. Inappropriate calpain activity is known to trigger degradation of desmin and α-spectrin in ischemic hearts [20–22], especially after an increase in intracellular Ca^{2+} levels due to myocardial infarction [21]. It should be noted that protein and mRNA levels of first calpain-2 and then calpain-1 increase after a myocardial infarction [23]. However, the degradation can be inhibited by synthetic calpain inhibitors [18].

Calpain has a broad range of substrate specificity [14, 18, 24]. A number of cellular proteins are calpain substrates which include calmodulin (CaM)-binding proteins (e.g., calcineurin, phospodiesterase1), endogenous inhibitors of Ca^{2+}-binding proteins (e.g., cain/cabin1), cytoskeletal proteins (e.g., spectrin, MAP-2), G-proteins, enzymes involved in signal transduction (e.g., PKC, IP3 kinase), membrane receptors (e.g., EGF receptor), and transcription factors [14, 15, 25–30]. Most calpain substrates possess hydrophilic residues such as proline, glutamic acid, aspartic acid, serine, and threonine (i.e. the PEST sequence) close to the cleavage site [24, 31, 32]. Calpain has been implicated in a number of pathological conditions and the enzyme has been proposed as a potential therapeutic target for a number of diseases including brain trauma, spinal cord injury, Alzheimer's disease, Parkinson's disease, subarachnoid hemorrhage, muscular dystrophy, cardiac ischemia, restenosis, thrombotic platelet aggregation, arthritis, and cataract. The implication of calpain in human disease has fueled interest in the discovery of calpain inhibitors as therapeutic agents [33].

3 Myocardial Involvement of Calpains

The involvement of calpains has been suggested in myocardial ischemia/reperfusion injury, myocardial stunning and cardiac hypertrophy [14, 34, 35]. Calpains can degrade several structural and cytoskeletal proteins such as tropomyosin, C-protein, desmin, troponins (T, C, I), filamin, nebulin, titin, alpha actinin and myosin [36–41]. Increased disruption of microtubules is associated with its action by increasing intracellular Ca^{2+} [42]. Calpains are activated by Ca^{2+} and are concentrated in the Z-disk, the site where myofibril disassembly begins [43]. The treatment of purified myofibrils with Ca^{2+} causes rapid and complete loss of Z-disks and partial

degradation of M-lines [42, 44]. These degradation processes during myocardial ischemia/reperfusion can cause altered properties of ion channels, disorganization of gap junctions, disorders in electrical conduction, impairment of the contractile machinery and myocardial dysfunction [37–41, 45].

Calpain cleaves inositol 1,4,5-trisphosphate receptor (InsP3R1), a ubiquitous intracellular Ca^{2+} release channel located on the endoplasmic reticulum (ER) membrane in selectively vulnerable cerebellar Purkinje neurons after *in vivo* cardiac arrest [46]. These findings indicate that calpain proteolysis of InsP3R1 generates a dysregulated channel which disrupts cellular Ca^{2+} homeostasis. Furthermore, it has been suggesting that Ca^{2+} may leak through the proteolyzed channel may act as a feed-forward mechanism to enhance cell death [47]. The regulation of cardiac hypertrophy in the heart is controlled by 2 transcription factors—nuclear factor of activated T-cells (NFAT) and NF-κB [48]. Impaired InsP3R-dependent Ca^{2+} release in Chromogranin-B (CGB) knockdown cardiomyocytes attenuates both NF-κB activation and production of BNP on angiotensin II (Ang II) stimulation [49]. It has been suggested that Ca^{2+}-dependent NF-κB activation in the heart is due to calpains [50]. The decreased activation of calpain in calpastatin transgenic mice impairs NF-κB activity and attenuates Ang II-induced hypertrophy, much similar to the impaired InsP3R-dependent calcium release in CGB knockdown cardiomyocytes on Ang II stimulation [49, 50]. These studies show that calpain is the missing link in cardiomyocyte Ca^{2+}-dependent NF-κB activation but may also be of crucial importance in the differential activation of Ca^{2+}-activated transcription factors such as NFAT and NF-κB [9].

Interestingly, recent studies have shown that conventional calpain activity is required for cardiovascular health. Recently, inhibition of endogenous calpain-1 by calpastatin overexpression, caused slow-progressing dilated cardiomyopathy in mice [51]. Similarly, knockout studies in mice showed requirement for conventional calpains in cardiac functions against haemodynamic stress which resulted in development of cardiac hypertrophy and cardiac dysfunction [52]. This result indicates that the activity of conventional calpains is essential to heart function and can provide protection against stress. Thus calpain is critical for the maintenance and degradation of cardiomyocyte.

4 Calpain: Calcineurin Signalling in Ischemia

Calcineurin (CaN) is a Ca^{2+}-CaM-activated serine-threonine phosphatase that is ubiquitously expressed and plays an important role in transducing Ca^{2+}-dependent signals [53]. CaN is a heterodimer composed of CaM binding catalytic subunit A (CaN-A) and a Ca^{2+}-binding regulatory subunit B (CaN-B) [54]. CaN effects Ca^{2+} signalling by regulating protein phosphorylation and participation in numerous cellular process including immune system responses, neuronal and muscle development, cAMP pathway, Na/K ion transport in nephron and cell cycle regression in lower eukaryotes [55]. Activation of CaN phosphatase activity requires both the binding of Ca^{2+} to CaN-B and Ca^{2+}-dependent binding of CaM to CaN-A. Studies

have suggested that the activation of CaN is also protease-dependent (Calpain, Trypsin, Chymotrypsin C) and can be irreversible both *in vitro* and *in vivo* [56]. Calpain removes the regulatory domain of CaN-A by site limited proteolytic truncation and activates phosphatase function without the requirement of Ca^{2+} and CaM [57, 58]. Calpain can also activate CaN via the cleavage of the endogenous CaN inhibitor cain/cabin 1, a protein that binds to and inhibits CaN with its C-terminal region [30]. Endogenous calpain-1 can constitutively proteolysis of CaN-A resulting in the formation of active CaN in human heart [59].

In heart, hypertrophic stimuli, such as angiotensin II, phenylephrine, and endothelin-1, can elevate intracellular Ca^{2+} levels and subsequent activation of CaN, which leads to dephosphorylation of the nuclear transcription factor NFAT, resulting in the induction of genes typical of cardiac hypertrophy. Calpain-induced activation of CaN has been observed in hypertrophied myocardium both *in vitro* and *in vivo* [56]. Animal models of myocardial hypertrophy demonstrated significant increase in calpain activity and calpain-mediated proteolysis of CaN-A, which produces a 48 kDa N-terminal fragment (residues 1–424) [60]. The 48 kDa N-terminal truncated form of CaN-A has been found *in vivo* in human hypertrophied myocardium [56]. Studies have shown that during ischemia and reperfusion, there is increased influx of Ca^{2+} into the cells, which can activate calpain-1 and calpain-2 [35, 61]. Ischemia followed by reperfusion (30 min each) displayed increased calpain activity in rat heart and subsequent calpain-2 mediated degradation of full-length CaN-A. Calpain-mediated cleavage created a 46 kDa truncated CaN-A and enhanced phosphatase activity of CaN [62].

It has been proposed that CaN and Ca^{2+}/CaM kinase II (CaMKII) can act as both substrate and activator for calpain [63]. Ischemia activates CaN which contributes to increase cytosolic Ca^{2+} concentration during early reperfusion through dephosphorylation of phospholamban. Phospholamban dephosphorylation prevents sarcoplasmic reticulum Ca^{2+}-ATPase activity and sarcoplasmic reticulum Ca^{2+} uptake [64]. It should be noted that very low levels of Ca^{2+} (<1 μM) cannot activate calpain. Limited proteolysis of CaN may increase its activity and can thus activate calpain through a feedback mechanism, thus contributing to the pathogenesis of myocardial disease. Increased expression of calpain and elevated activity of CaN was observed in the myocardium of patients with congestive heart failure [65]. Therefore, calpain-CaN-A signaling is a critical contributor to the pathogenesis of ischemic myocardium (Fig. 1).

5 Calpain: Phosphodiesterase 1 Regulation in Heart

Cyclic nucleotide phosphodiesterases (PDEs) are a class of ubiquitous enzymes that catalyses the hydrolysis of phosphodiester bonds and specifically inactivate the intracellular messengers cAMP and cGMP in a compartmentalized manner. PDEs constitute of 11 gene-related families of isozymes (PDE1 to PDE11) [66]. As downstream receptors, PDEs play a major role in controlling the signalosome at various levels of phosphorylations and protein/protein interactions [67]. Due to the

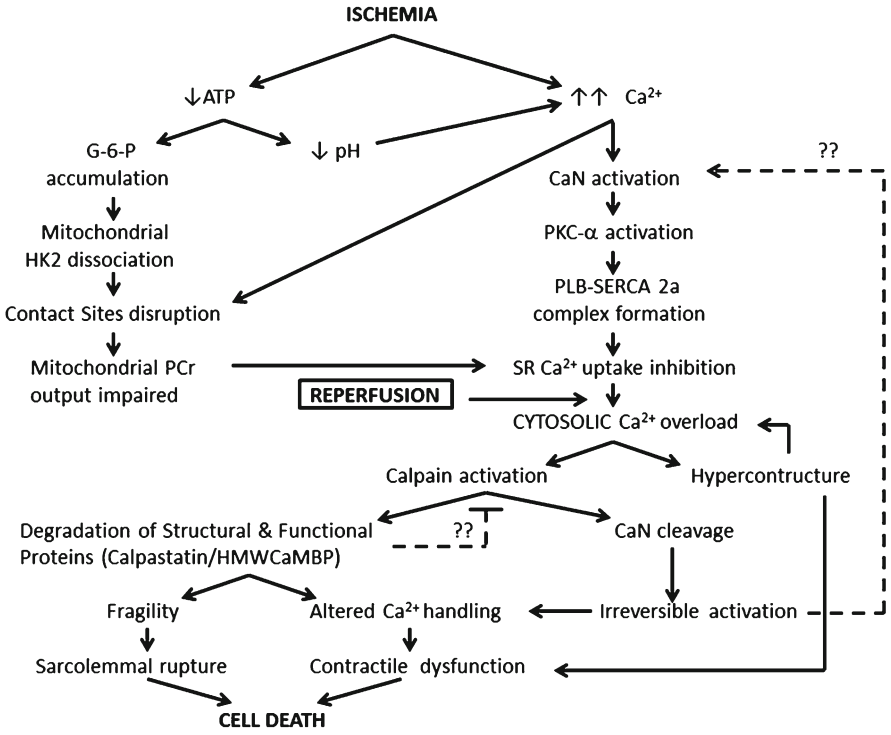

Fig. 1 Schematic representation of calpain-CaN signaling in myocardial ischemia. The *dotted lines* with query symbols represent hypothesised pathways. G-6-P indicates Glucose-6-Phosphate; HK—Hexokinase; PCr—phosphocreatine; HMWCaMBP—High Molecular Weight Calmodulin-Binding Protein; CaN—Calcineurin; PKC-α—Protein Kinase C—α; PLB—Phospholamban; SERCA—Sarcoendoplasmic Reticulum Calcium Transport ATPase

multiplicity of isozymes, their various intracellular regulations and their different cellular and subcellular distributions, PDEs represent interesting targets in intracellular pathways. PDEs have an important role in the regulation of inotropic mechanisms in the human myocardium. In the myocardium, at least six different PDE families have been described, including PDE1, 2, 3, 4, 5, and 8. PDE1, 2, and 3 are dual-specific PDEs that can hydrolyze either cAMP or cGMP, whereas PDE4 and 8 specifically hydrolyze cAMP and PDE5 specifically hydrolyzes cGMP. Within these PDE families, over a dozen of different isoforms are expressed, either as products of different genes or as products of the same gene through alternative splicing and/or by differential use of translation starting sites [68–70].

CaM-stimulated cyclic nucleotide phosphodiesterase (PDE1) is one of the key enzymes involved in the complex interaction between cAMP and Ca^{2+} second messenger systems and is essentially expressed in a nonmyocyte fraction of cardiac tissue [71, 72]. Some PDE1 isozymes have similar kinetic and immunological properties, but are differentially regulated by Ca^{2+} and CaM and can be distinguished by

various pharmacological agents [72]. The regulation of PDE1 by calpain is involved in neuronal and cardiac function. Studies have suggested that limited proteolysis by calpain could be an alternative mechanism for the activation of PDE1 [73]. Our group had previously shown PDE1 activity, expression and effect of calpain in the rat model *in vitro* of cardiac ischemia-reperfusion [74].

Proteolytic cleavage of PDE1A2 by calpain-2 generated a 45 kDa immunoreactive fragment, which is an active CaM-independent form [75]. The presence of CaM in the proteolytic reaction did not have any effect on PDE1A2 activity, suggesting that the interaction between CaM and PDE1A2 does not alter substrate recognition by calpain. High molecular weight CaM-binding protein (HMWCaMBP), which was discovered in our laboratory [76], is homologous to calpastatin [77] an endogenous inhibitor of calpains and did not alter PDE1A2 activity but did inhibit calpain-mediated proteolysis. A CaM overlay using biotinylated CaM revealed that the 45 kDa fragment does not contain the CaM binding domain [75]. Furthermore, the phosphorylation of PDE1A2 by cAMP-dependent protein kinase and treatment with calpain-2 resulted in generation of the 45 kDa fragment, suggesting that phosphorylation does not protect PDE1A2 from calpain-2 action. In contrast, a previous study on connexin-32 indicated that its phosphorylation by protein kinase C prevents proteolysis by calpains [78]. It has been shown that two serine residues of PDE1A2 are phosphorylated by cAMP-dependent protein kinase, at serine 112 and serine 120 [79]. Our results suggested that the calpain cleavage site could be distant from the phosphorylation site [75]. The PEST regions have been suggested to be recognized by specific proteases, particularly the Ca^{2+}-dependent cysteine proteases, calpains [31, 32]. However, the cleavage site for calpains rarely resides in the PEST motif [80], although in some calpain substrates, PEST sequences are located near the cleavage site. PDE1A2 has a single PEST motif (residues 73–94), and calpain cleaves PDE1A2 after residue 126, which is 32 residues away from the PEST motif [75].

PDE1A2 is a 530-residue polypeptide, a homodimer that is Nα-acetylated, and is composed of separate catalytic and regulatory domains [81–83]. From the N-terminus, the CaM binding site is located from residue 23 to 41, the PEST motif from residue 73 to 94 and the phosphorylation site from residue 110 to 120, respectively. The catalytic domain encompasses an approximately 250-residue sequence (139–446) which is conserved among PDE1 isozymes of diverse size, phylogeny and function [84]. Our analysis revealed that the molecular weight of this domain was 45 kDa, suggesting that the fragment is comprised of residues 139–530, when the enzyme is converted into a CaM-independent form (Fig. 2). The most probable site for calpain cleavage could be between residues 120 and 138 and this was confirmed by the N-terminal sequence analysis of the 45 kDa fragment, which showed that the calpain cleavage occurs between residues Glutamine 126 and Alanine 127. The findings suggest that the proteolysis of PDE1A2 by calpain-2 results in a CaM-independent form, which in turn could decrease the intracellular levels of cAMP [73, 75].

In cardiac ischemic-reperfused rat model, ischemia-reperfusion injury to myocardium alters the activity of heart PDE1A1 while having minimal effects on cAMP concentrations [74]. The total PDE1A1 activity and expression in rat heart was

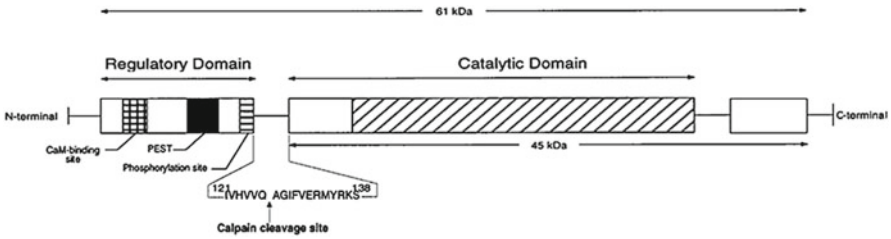

Fig. 2 A representation of the PDE1A2 showing the cleavage site by calpain-2. The regulatory and catalytic domains are shown. The *lined*, *black*, and *checkered boxes* represent the CaM-binding domain, PEST, and phosphorylation site followed by conserved catalytic domain (hatched area), respectively. The *arrow* indicates the calpain-2 cleavage site between residues Glutamine 126 (Q) and Alanine 127 (A). Reproduced with permission [75]

largely unchanged under various conditions of ischemia and reperfusion. However, the basal activity which is independent of Ca^{2+}/CaM, increased with increasing degree of ischemia reperfusion. Pre-treatment of rat hearts with cell permeable calpain inhibitor (ALLM, N-Acetyl-Leu-Leu-Methional) provided a protective effect on PDE1A1 activity as total PDE1A1 activity in this group was found to be 97 % of the control group. However, protein expression of PDE1A1 was not altered in the ischemic and reperfused rat hearts. The protective effect of calpain inhibitor upon PDE1A1 in rat heart appears to be due to a decrease in calpain-mediated proteolysis of PDE1A1. In vitro incubation of PDE1A1 with calpain-1 and 2 demonstrated that the PDE1A1 isozyme was susceptible to proteolysis by the calpains.

Thus, calpain-2 was capable of generating CaM-independent forms of PDE1A2 following proteolysis [75]. Heart PDE1A1 was susceptible to proteolysis by both calpain-1 and 2 [74]. Such a pathway allows decoupling of the Ca^{2+} signal from PDE1A1 under sustained conditions of increased intracellular Ca^{2+}. Variations in PDE1A1 activity did not affect the total cAMP concentrations in rat myocardium. The generation of a CaM-independent PDE1 during ischemic events may therefore allow for the maintenance of cAMP levels in the case of Ca^{2+} level derangements following ischemia [85]. During reperfusion, an increased uptake of Ca^{2+} has been observed in myocytes, which would be capable of activating calpains [86]. These activated calpains could then proteolyse PDE1A1 and irreversibly activate it, preventing the potentially toxic accumulation of cAMP in myocytes. While cAMP is necessary for the normal and effective cardiac function [87], it has also been shown to have arrhythmogenic effects, which was studied by the use of PDE inhibitors [88]. By preserving the degradative portion of the cAMP pathway via generation of CaM-independent PDE, a physiologic mechanism of maintaining cAMP in acute and chronic myocardial injury exists, thereby preventing further myocardial damage [73]. Thus, a dynamic interaction between calpains and PDE1A1 in an ischemia-reperfusion model of the heart may allow for the maintenance of physiological concentrations of cAMP during periods of hypoxic injury to the heart.

6 Calpain Inhibitors

Calpain activity is crucial for proper cardiac function including repair [51, 52]. However, increased conventional calpain activity is an aggravating factor in cardiovascular diseases and in many conditions the damage can be reduced by inhibiting calpains [89]. The search for calpain inhibitors has been an ongoing process following the discovery of the enzyme over 40 years ago and has been described well by various authors [18, 19, 24, 33]. General protease inhibitors such as EDTA and EGTA (Ca^{2+} chelators), iodoacetic acid, 5,5′-dithiobis(2- nitrobenzoic acid), N-ethylmaleimide, mersalyl, isocoumarins, and diisopropyl phosphorofluoridate were among the first inhibitors of the enzyme to be identified. Besides these, a number of peptidyl calpain inhibitors derived from mammalian, plant, and synthetic sources have appeared in the literature. Current knowledge about calpain substrate specificity and the nature of the active site of the enzyme have made possible the development of potent and in some cases selective calpain inhibitors [24]. The use of calpain inhibitors to prevent ischemic apoptotic death in several cell types, such as the heart, brain, liver and renal cells, has shown promise because of the reduction in the extent of the lesions and improvement in the recovery of normal tissue functions [19, 24, 33]. Calpain inhibitors have demonstrated efficacy in animal models of calpain related diseases, but progression of the inhibitors into clinical trials has been hampered partly due to lack of calpain isoform selectivity and the general reactivity of the inhibitors. Therefore, efforts should be directed towards the discovery of compounds devoid of these problems. Exploration of compounds that bind to allosteric sites of the enzyme may circumvent these problems and afford new drug leads. Majority of these inhibitors are active site directed reversible or irreversible agents with limited selectivity for calpain over other cysteine proteases [33]. Calpain inhibitors can be broadly classified into macromolecular (calpastatin and HMWCaMBP) and micromolecular (synthetic molecules and non-peptide molecules).

The current review focuses on macromolecular inhibitors of calpain since; calpastatin is the most specific calpain inhibitor on record. Calpain activity is controlled *in vivo* by calpastatin, a protein encoded by the calpastatin gene [13, 14, 19]. Calpastatin is a multi-headed endogenous polypeptide that specifically inhibits both major isoforms of calpain but does not act on any other proteases like papain and cathepsin B [90]. The specificity of calpastatin is determined by the simultaneous binding of three calpastatin subdomains to domains II, IV and VI of the heterodimeric calpains [19, 91]. The polypeptide consists of an N-terminal domain (domain L) and four repetitive domains (repeats 1–4 of about 140 amino-acid residues). All four repeats have similar inhibitory activity against calpains I and II but domain L is devoid of inhibitory action. Each inhibitory domain of calpastatin has three subdomains, A, B, and C; A binds to domain IV and C binds to domain VI of the calpains [13, 91].

Calpastatin penetrates poorly into cells and overcoming this barrier has generated significant interest. Truncated forms of calpastatin such as the 27-mer peptide derived from exon 1B of calpastatin is a very potent and selective inhibitor of calpain but penetrates poorly into cells [92]. To overcome this drawback, synthetic peptide derivatives based on repeat region of calpastatin were developed and was

observed to inhibit calpain. These reports demonstrate that enhanced penetration of calpastatin or its derivatives in cells can be achieved by appending the protein to a cell-penetrating peptide such as penetratin or poly arginine peptide [24, 33, 93]. A recent review on calpain inhibitors clearly says that further work is required to study if amphipathic cell penetrating peptides such as Tat, transportan, pVEC and MAP can also facilitate penetration of calpastatin and related peptides into cells, without loss of potency and selectivity for calpain [33]. Other polypeptide inhibitors of calpain are α2- macroglobulin [94], Growth arrest-specific protein 2 (GAS2) [95], Spectrin fragments (in brain) [96], and the heavy chains and derivatives of L- and H-kininogen [97, 98]. In cardiac tissue, HMWCaMBP has been identified as a homologue of both calpastatin 1 and 2 [77]. Calpastatin has been observed to regulate the cleavage of the Cdk5 activator p35 to p25 by calpain [99].

7 Possible Advances in Future

Calpain was first identified in 1964 and since then, work has been mainly directed towards understanding physiological significance, activation, substrate recognition and inhibitors [100]. Nevertheless, several questions still remain unanswered. The most important question is how does calpain work? The mechanism of calpain activation has been resolved using its 3-D structure [13]. "Proteolytic processing" of substrate by calpain results in the formation of a un-degraded product (the proteolyzed substrate) which in contrast, is degraded by both proteasomes and autophagy [100]. Interestingly, calpains are directly involved in substrate recognition, whereas proteasomes and autophagy act on substrates "tagged" by other systems, such as ubiquitination and selective/non-selective autophagosomes. Hence, it is really interesting to understand how calpain works in body since, knockdown models of calpain have already demonstrated the relevance of calpain in homeostasis.

According to Ono *et al.* [100], the major research avenues on calpains in future are

1. Structure based identity
2. Parameters involved in activation
3. Substrate specificities

It is expected that systematic characterization of calpain-mediated proteolysis will enable the use of thought-experiments as this approach had been attempted with caspase system [101, 102]. Combining systemic characterisation with "ordinary" experiments could be effective in identifying novel calpain functions. Understanding of species-specific activation mechanisms would stimulate the elucidation of physiological functions, and *vice versa* [100]. Difficulties in recombinant calpain production hinder generation of complete crystal structures. Lack of complete structures prevents one to be completely confident about any aspect of calpain activation [103]. Therefore, the structural and functional characteristics of calpains have to be studied in detailed for obtaining the goal of homeostasis after cardiac ischemia.

8 Conclusion

This review summarizes the research in the field of calpain biology with respect to cardiac system. The significance of calpain-CaN signaling and PDE1 cleavage by calpain in cardiomyocytes following ischemia has been recognised. It is time that the important findings from biochemical studies are verified *in vivo*. It is expected that the technical advances will pave way in understanding the diverse and complex nature of the calpain family since, many questions still exist within the field of calpain research. Ono *et al*. [100], summarised their review on calpains—"This is the way that calpain research has been, and always will be, carried out."

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Exercise and Matrix Metalloproteases in Health and Disease: A Brief Overview

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Abstract Systematic exercise plays a great deal with health for people to improve and/or prevent many diseases such as hypertension, coronary heart disease and diabetes. However, strenuous exercise markedly increases expression and activation of matrix metalloproteases and thereby causes changes in the regulation of skeletal muscle and tendon functions, immune response, aging and angiogenic processes. This review provides information on some cellular and molecular responses that underlie the prophylactic effects of exercise in some pathophysiological conditions including age related diseases involving matrix metalloproteases.

Keywords Exercise • Matrix metalloproteases • Tissue inhibitors of metalloproteases • Aging • Angiotensin • Endostatin • Immunity • Gene expression

1 Introduction

Matrix metalloproteases (MMPs) are a family of highly homologous Zn^{2+} endopeptidases that collectively cleaves most, if not all, of the constituents of extracellular matrix (ECM). MMPs in the circulation are thought to modulate the activation of growth factors, cytokines [1] and angiogenesis, thereby facilitating physiological adaptations to exercise training [2–4]. The MMP family of enzymes contribute to both normal and pathological tissue remodeling [5, 6]. Each MMP targets a specific substrate, thus the appropriate MMP is released in a time and location specific manner to orchestrate membrane remodeling and adaptation [7, 8].

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Regular exercise is of great importance for public health. It has been shown to be effective in the prevention and therapy of many wide spread diseases such as hypertension, coronary heart disease and diabetes [9, 10]. However, exhaustive exercise causing inflammatory responses lead to significant change in the activity of MMPs such as MMP-9 and MMP-2. The heavy activities and deleterious effects that activate MMP-9 and MMP-2 are indicators of inflammatory conditions, which eventually cause degradation of extracellular matrix leading to the incidence of diseases, for instance, arthritis [11].

Many aspects of how the prophylactic effects of exercise on chronic diseases are mediated remain unclear [12, 13]. One important way to improve our understanding of these beneficial effects is to gain insight into the cellular and molecular responses to exercise.

2 Skeletal Muscle and Exercise

Certain stimuli, particularly those that induce high levels of mechanical stress by high impact exercise activate the local production of MMPs in skeletal muscle [14]. Serum concentrations of MMPs are reported to peak within a relatively short time period following a single bout of exercise. MMPs in the circulation are thought to modulate the activation of growth factors and cytokines through degradation of their precursors, binding proteins and inhibitors [15, 16]. Strenuous exercise, especially fast speed running, is known to cause intra- and extra-myofibrils damage [15, 16]. High intensity exercise increased both mRNA and protein levels of MMPs [3, 17]. It is generally accepted that MMPs function in skeletal muscle to process extracellular matrix (ECM) proteins, thereby regulating matrix degradation and repair, while those released into the circulation facilitate angiogenesis [18, 19].

Skeletal muscle fibre possesses a high degree of functional and structural plasticity and is capable of responding rapidly to changes in contractile activity [20]. The diaphragm is a unique skeletal muscle that is considered to be two muscles in one. This fact is based on anatomical and functional differences between the coastal and crural regions [21] that contain different composition in muscle fibre types. The crural diaphragm is dominated by type IIb fast twitch muscle fibres, whereas the coastal diaphragm contains equally type I (slow twitch) and type IIb (fast twitch) fibres [22]. Type IIb muscle fibres are more susceptible to ECM degradation than type I muscle fibres during exercise [12]. Moreover, a degree of muscle tissue change is fibre type specific and appears to be more pronounced in type IIb fibres following exhaustive exercise, which suggests a higher protein degradation in fast twitch (type IIb) than in slow twitch muscle fibres (type I) [12]. Thus, type II muscle fibres are more susceptible to exercise overuse than type I muscle fibres, and fast fibres are more responsive to exercise induced changes in MMP expression [12].

Superoxide dismutase (SOD) level significantly decreased in the crural diaphragm muscle of rat about a month of fast speed running; whereas it remained unchanged in the coastal diaphragm muscle during the period of running [12].

The expression of MMP-2 was found in both fast and slow running groups; however, it was particularly prominent in fast twitch muscle [12, 15]. High intensity endurance exercise increases MMPs such as MMP-2 and MMP-9 expression, whereas low intensity endurance exercise did not alter MMP-9 and MMP-2 activities in the skeletal muscles such as gastrocnemius (back part of lower leg), quadriceps (large muscle group on the front of the thigh) and soleus muscles (closely connected to gastrocnemious) [12, 13, 15]. In contrast, high intensity exercise increases both mRNA and protein levels of MMP-2 in skeletal muscle containing a high percentage of fast type IIb fibres [19]. Thus, high intensity exercise is required to promote the expression of MMP-2 in skeletal muscles and that the influence of exercise on MMP-2 expression has been found to be dominant in the muscle containing a high percentage of fast fibres [12, 15]. Thus, based on the concept that high intensity exercise in untrained animals' results in significant muscle remodeling [23], Carmeli *et al.* [12] hypothesized that high intensity exercise would promote markedly the expression of both MMP-2 and MMP-9 in skeletal muscle, whereas low intensity results in limited changes in muscle levels of MMP-2 and MMP-9 [19, 23].

Treadmill exercise can serve a model to demonstrate different damage pattern in the two separated part of diaphragm muscle during exhaustive exercise. The integrity and the composition of ECM were affected considerably through expression of MMPs, for example, MMP-2 and thereby cause change in the collagen synthesis particularly in type IIb muscle fibres [19]. There are potentially three explanations for the differences in MMP-2 expression between the muscle fibre types. First, in rat skeletal muscles, type IIb fibres are at least twice as big as type I fibres, which probably explains a higher volume of collagen; therefore, white fibres (type IIb, fast twitch) requires more MMP-2 to maintain its integrity than red fibres (type I, slow twitch). Secondly, white fibres elicit better muscle plasticity than red fibres and show faster adaptation to exercise training. Under intensified training, fast fibre may undergo transition to intermediate muscle fibres (type Ia) with corresponding changes in ECM compositions [12]. Following the fast and prolong running, the type II muscle fibres are thought to be more susceptible to oxidative stress in order to produce a greater aerobic energy [13]. And, thirdly, the diaphragm differs from locomotor skeletal muscle in adaptation to exercise [24].

The recruitment of coastal fibres increases from rest to low intensity of exercise. During intense exercise, the coastal region reaches a plateau in motor unit recruitment before meeting the ventilatory demand. This suggests that type IIb fibres are not recruited during normal ventilatory behavior. Therefore, in contrast to type I, high intensity performance of type IIa provides a trigger for type IIb intra-and extracellular adaptations [12, 13, 24].

Currently limited information exists regarding the effects of exercise training on MMPs in skeletal muscle. Studies have been made to compare the ways of activity of two types of proteinases: MMP-2 and MMP-9 during a patho-physiological process in the trained subjects (with long term adaptation to exercise) and untrained or non athlete persons (without adaptation to exercise). MMP-2 and MMP-9 activities in athletes have been found to be significantly increased immediately after exhaustive exercise and significantly decreased in the next day; while in non-athletes,

MMP-2 and MMP-9 activities were also increased immediately after exhaustive exercise and that was similar to athletes, but did not show any discernible decrease in the activities of the enzymes in the next day after exercise [1].

3 Angiotensin Blockers and Exercise

Exercise training is emerging as an important complementary intervention in heart failure [25, 26]. Exercise has been shown to enhance aerobic capacity, attenuates left ventricle (LV) dilation, regress cellular hypertrophy and improves cardiomyocyte contractility and myofilament function [27, 28]. These beneficial effects could be due to attenuation of renin-angiotensin system (RAs), which is known to improve cardiac remodeling [29, 30]. Indeed, exercise training normalized circulating RA system in patients with heart failure [31].

The impact of exercise training on factors contributing to myocardial fibrosis is not clearly known. It has been suggested that exercise training induced improvement of cardiac function may be due to marked decrease of myocardial fibrosis and remodeling [30]. So, combination of AngII blockade and exercise training on myocardial remodeling and function after myocardial infarction was assessed by many investigators. Studies on both gene and protein levels of MMP-2, MMP-9 and TIMP-1, angiotensin converting enzyme (ACE) and angiotensin receptor-1 (AT-1) after myocardial infarction (MI) suggest that exercise training improves factors involving post-MI fibres, reduce collagen content and thereby attenuates deleterious cardiac remodeling and preserve cardiac function. Such beneficial effects have been found to be further accentuated by angiotensin II type I (AT1) receptor blockers e.g., losartan [30].

MMPs and TIMPs are the key elements involved in matrix degradation and contribute to myocardial remodeling after MI. Increased MMPs expression or decreased TIMPs expression could result in enhanced proteolytic activity and degradation of ECM molecules [32]. Webb *et al.* [33] demonstrated that TIMP-1 level was high at day 1 post-MI and remain substantially elevated through 6 months in patients with MI. The elevated TIMP-1 level may contribute to the accumulation of collagen content in the infarcted heart, leading to myocardial fibrosis [34]. Interestingly, TIMP-1 has been shown to be increased significantly about 2 months of post MI. Both exercise training and losartan attenuates TIMP-1 expression at both gene and protein levels. Exercise training and losartan treatment in combination after MI has been shown to reduce the TIMP-1 expression, improves the balance between MMPs and TIMPs and enhance the proteolytic activity of post MI, which subsequently decreases collagen accumulation. This leads to decrease in cardiac stiffness, preserve left ventricular systolic pressure and ventricular performance, and reduce left ventricular end diastolic pressure significantly in the late phase of post MI [30]. Although the exact mechanisms of post MI exercise training induced beneficial effects on myocardial remodeling are not fully elucidated, several studies have suggested that the effect of exercise training may be due to increased baroreflex

sensitivity, reduced sympathetic activity and enhanced vagal tone [30, 35, 36]. In addition, a reduction in circulating AngII by exercise training may act favorably on baroreflex control of sympathetic activity [30, 37, 38]. Interestingly, early decrease of TIMP-1 in the infracted heart coincides with collagen degradation in the necrotic myocardium, whereas the subsequent increase of TIMP-1 in the infarct heart contributes to collagen accumulation at the late phase of post MI remodeling [39].

Recent research suggest that a decrease in the expression of TIMP-1 and ACE levels, and also a decrease in AT1 receptor numbers are associated with the mechanism by which losartan and exercise could attenuate fibrosis and preserve cardiac function [29]. Exercise training and/or AngII receptor blockage after MI has been suggested to play an important role in cardiac remodeling by attenuating TIMP-1 expression, improving the balance between MMPs and TIMPs, attenuating ACE and AT1 receptor expression, and thereby decreasing collagen content [30]. These improvements, in turn, attenuate myocardial fibrosis and cardiac stiffness and preserve post MI cardiac function [40, 41].

4 Immunity and Exercise

TNF-α level increases within an hour after exhaustive exercise and that has been shown to stimulate activities of MMPs. Cytokines such as TNF-α and interleukins, for example, IL-8 levels in blood of mononuclear cells have been shown to be increased in response to strenuous exercise, which remain elevated, respectively, at 2 h for IL-6 and up to 24 h for IL-1β and TNF-α after exercise [42]. MMP-1, for instance, has been demonstrated to be involved in lymphocyte trafficking [42].

Exercise has been shown to be an important regulator of immune cells and their functions, therefore, white blood cells (WBCs) have been chosen as target cells [43]. In circulating WBC's, exercise induces upregulation of MMP-9, which breaks down native collagen as well as other extracellular matrix molecules [44]. There are a number of reports on an increase in collagen degrading activity as well as release of MMP-9 in muscle, interstitial fluid and serum especially after strenuous exercise [23]. The role of MMPs in haematopoietic system during exercise involves the mobilization of progenitor cells [45]. Studies in rhesus monkeys suggest involvement of MMP-9 in IL-8 induced mobilization of hematopoietic progenitor cells (HPCs) via cleavage of matrix molecules to which the stem cells adhere [46]. Recently, it has been suggested that activation of MMPs, for instance, MMP-1 in peripheral blood mononuclear cells (PBMCs) during exercise may be involved in lymphocyte trafficking and in the recruitment of progenitor cells from bone marrow [11, 47]. Given that exercise regulates function of immune cells, role of PBMCs in the physiological changes associated with strenuous exercise has been suggested to be an important aspect of exercise physiology [48]. Changes in the expression of a group of genes within the leukocytes may serve as surrogate markers for systemic or local modifications induced by exercise [49, 50]. The response of leukocytes to

exercise on the expression of genes is known to some extent [51, 52]; however, complete lists of genes that are differentially expressed have not yet been fully explored. The regulatory mechanisms associated with the expression of the genes are also currently unknown.

Inflammatory responses induced increase in cytokines and subsequently MMPs levels have been shown to be redistributed between the lymphoid and non lymphoid organs, which cause mobilization of HPCs from bone marrow. These processes have been related to exercise induced stress [45, 53]. MMPs, for instance, MMP-1 is involved in the enhanced peripheral invasion and migration to tissues of natural killer cells (NKs). An increase in the production of MMPs from stimulated NK cells plays an important role in the facilitation of lymphocyte trafficking and in the accumulation of lymphocytes in tissues during pathophysiological processes [54]. Cytokines stimulate the expression of multiple MMPs in lymphocytes. MMPs, for example, MMP-1 produced by cytokines, for example, IL-8 stimulated NK cells play a role in the degradation of matrix collagens [55, 56].

Recent reports have suggested that redistribution of leukocytes is a fundamental regulatory mechanism of the haematopoietic system that alters lymphocyte counts during exercise [53]. The ability of immune cells to migrate appears to be closely regulated by molecules such as cytokines and MMPs, for instance, MMP-2, which are mediated through receptors of the integrin family members [57]. Although, the exact mechanism by which lymphocytes invade tissues is not completely understood, this migration seems to be regulated by distinct pathways involving mitogen activated protein kinases (MAPKs). Goda *et al.* [54] demonstrated that MMP-1, for instance, is produced by the cytokines, for example, IL-8 stimulated NK cells and that is associated with $\alpha_2\beta_1$ integrin, indicating that integrins could play a role in the immobilization of MMP-1 on the cell surface. The binding of MMPs to integrins could be the crucial step for promoting lymphocyte migration given the fact that disruption of their association decreases the migratory activity of cells [58]. Integrins are also known to be involved in signaling pathway(s) of the Rho family of GTPase that has been shown to be associated recently with modulation of the expression of IL-8 and MMP-1, which are induced upon inhibition of the MAPKs pathway [57].

Inflammation and the resulting inflammatory response for athletes indicated that MMP-2 and MMP-9 were not return to normal levels even 24 h after exhaustive activity; while for non athletes, the response was even much weaker [11]. There is evidence that exercise stress works an inflammation like reaction on the immune system with the activation of both pro-inflammatory and anti-inflammatory pathways, which is dependent on exercise intensity and duration [59]. Therefore, acute exhaustive exercise is expected to transiently decrease the individual's immune competence, while moderate exercise has an anti-inflammatory effect with improved anti-infections capability [60]. There is growing evidence that the immune system may serve as an important physiological indicator for a person's individual activity to recover from workload stresses [11, 60]. However, the overtraining syndrome, a condition of long term decrement in performance capacity due to continuous training loads, is based on derangement of cellular immune regulation [11, 60].

5 Tendon and Exercise

Tendon has been considered as a model to explore sex differences in mechanical and metabolic properties of connective tissue [23, 61–63]. Tendon is a metabolically active structure that transmits force from muscle to bone for mechanical movement. ECM of tendon mainly consists of collagen and elastin fibres and is surrounded by an aqueous matrix of proteoglycans, glycosaminoglycans and glycoproteins [61, 62]. Mechanical properties and metabolism in tendon have been shown to be different in men and women [63, 64]. The elastic modulus is significantly decreased in women compared with men possibly leading to an inefficient matrix for force transfer between muscle and bone [62, 65]. In addition, cross-sectional structure revealed that tendons of habitually trained women have the same size as those of untrained women, whereas men's tendon assumed hypertrophy with exhaustive exercise training [65, 66]. Tendons collagen synthesis at rest and after an acute bout of exercise is significantly lower in women than in men [65, 66]. The increased risk for connective tissue injury in women compared with men may be related to structural and regulatory expression differences in tendon [61, 63].

Tendon is mainly composed of collagen and an aqueous matrix of proteoglycans that are regulated by MMPs and TIMPs. It has been demonstrated that collagen type-I, -III and MMP-2 mRNA expressions in patellar tendons were downregulated 24 h after exhaustive exercise. Women had higher mRNA expression of MMP-2 than men after 24 h of exercise [67]. This suggests that sex plays a role on the structural and regulatory mRNA expression of tendon. However, details of the mechanisms by which sex influence tendon metabolism and its mechanical properties is currently unknown.

6 Aging and Exercise

Regular exercise effectively improves heart function in both young and older populations [68, 69]. Exercise training improves maximal cardiovascular work capacity by increasing stroke volume and cardiac output [65, 68, 69]. Conceivably, exercise training in the aging population may reduce accumulation of connective tissue. It has been suggested that exercise training may attenuate collagen content in the aging heart [65, 69]. The ability of exercise training to attenuate diastolic dysfunction and collagen cross-linking has recently been cited [70]. Collagen cross linking (hydroxylysyl pyridinoline) of left ventricle (LV) free wall was demonstrated to be significantly lower in old trained rats compared with their sedentary counterparts [70, 71]. However, potential pathways by which exercise training ameliorates fibrosis in the aging heart are not understood. Collagen fibrosis with aging is progressive and associated with reduced cardiac contractility and risk of heart failure. Elevation of fibrotic connective tissue could lead to decreased cardiac compliance and impaired diastolic function, thereby increasing the risk of heart failure observed with aging [65, 72].

Collagen of the basement membrane is a typical substrate for MMP-2; though fibronectin, a structural component of the sarcolemma, can also be cleaved by MMP-2 and other MMPs that are inhibited by TIMP-1 [73, 74]. Cleavage of the ECM components may cause structural change that allows for adaptive processes such as satellite cell migration and fusion to the myofibre, or it may play a bioactive role in regulating cell proliferation and differentiation [73–75]. The MMPs, for instance, MMP-2; the MMP inhibitor, TIMP-1; and the ECM proteins, for example, fibronectin have been identified as factors whose expressions are altered by aging and exercise [73–75]. The relationship between expression of these factors and strength gain suggests that cleavage of muscle ECM or altered production thereof is an important process during training adaptation [73, 76].

Exercise training reduced fibrosis when visualized with collagen type I positive staining and novel imaging in the hearts of old rats [77]. Exercise training has also alleviated age-related downregulation of active MMPs, which cleaves fibronectin in sarcolemma and connective tissues [74, 75]. Age is known to up regulate TIMP-1 expression [78]. The inhibitory effect of TIMP-1 on MMP activation in aging has been found to be virtually abolished by exercise training [77, 79]. Recent reports have suggested that habitual exercise training attenuates age associated collagen accumulation and fibrosis through signaling pathway(s) that reduces MMP deregulation through TIMP-1 [69, 74, 77, 80].

Lubos *et al.* [34] reported that human heart failure is associated with a large up regulation of TIMP-1. However, ischemia reperfusion acutely activates MMPs [77, 79]. These observations can be explained by differential regulation between aging and hypertension, where MMP activity decreases with aging and increases with hypertension [77, 79]. Apparently, impaired turnover of ECM proteins could be an important contributory mechanism for accumulation of fibrotic tissue in the hearts due to aging [77].

Exercise training upregulates MMPs such as MMP-1, MMP-2, MMP-3 and MMP-14, thereby mitigating age related reduction in the expressions of MMPs. There are highly novel findings that are consistent with signaling pathways involving MMP regulation eliciting the protective effects of exercise training against remodelling, collagen accumulation and fibrosis. MMP-1, MMP-2, MMP-3 and MMP-14 serve as collagenases and degrade a host of ECM proteins including aggracan (MMP-1, -12 and -3), fibronectin (MMP-2, -3, -14), laminin (MMP-2, -3, -14) and gelatin (MMP-1, -2, -3, -14) [72, 81, 82]. Elevation of TIMP-1 observed with aging appeared to be consistent with upstream suppression of active MMPs such as MMP-1, -2, -3, and -14 [65, 72, 81, 82]. Additionally, exercise training in aged person substantially reduced TIMP-1 along with upregulation of MMP-1, -2, -3 and -14 levels [65]. Given that TIMP-2, TIMP-3 and TIMP-4 are not responsive to exercise training, the TIMP-1/MMP pathway has been suggested to be crucial for exercise-mediated protection against age related fibrosis [78]. Currently, TIMP-1 is considered as a target candidate for the beneficial effects of exercise [65, 78].

7 Endostatin and Exercise

Several members of the MMP family have been shown to be associated with ECM remodeling and these proteases mediate the proteolytic release of the angiostatic factor, endostatin from collagen [83]. Endostatin is a ~20 kDa –COOH terminal fragment of collagens [84]. In a recent study, plasma endostatin concentration has been shown to be increased in response to a single bout of exercise [85]. Regardless of the organ, time and/or the mechanisms responsible for the changed plasma level of endostatin, exercise regulates circulating levels of angiogenesis regulatory factors that could influence angiogenesis-dependent processes in the vascular system, for example, atherosclerosis [86].

Endostatin has been suggested to act as an anti-angiogenic factor by inhibiting vascular endothelial growth factor (VEGF) induced endothelial cell migration and proliferation [87, 88] and by the induction of endothelial cell apoptosis [86, 89]. However, Schmidt *et al.* [90] demonstrated that both pro-angiogenic and anti-angiogenic effects of endostatin could occur in a dose dependent manner. Interestingly, a recent study demonstrated that endostatin evokes vascular relaxation by increasing the cytosolic NO production [91], which suggests that endostatin regulates the local blood supply during strenuous physical activity.

In the angiogenic process, MMPs play critical roles in regulating endothelial cell adhesion, proliferation and migration and can, therefore, affect neovascularization [83, 92]. MMPs seem to have bilateral functions in angiogenesis. Firstly, in their active form, these enzymes facilitate the degradation of ECM components and neovascularization; and secondly they indirectly inhibit the angiogenic process of endothelial growth by generating anti-angiogenic growth factors and endostatin [87, 93]. In skeletal muscle, angiogenesis occurs as an adaptation to increased work requirements [87]. Exercise studies have focused on stimulatory (angiogenic) and inhibitory (angiostatic) factors in skeletal muscles [86, 94]. Role of angiostatic factors in human skeletal muscle and their change during exercise is an interesting aspect of exercise physiology.

8 Conclusion and Future Direction

Members of the MMP family are present in the skeletal muscles of healthy humans. MMP-9 is induced by a single bout of exercise, presumably by post translational activation and also by an increase in mRNA expression. In contrast, MMP-2 and MMP-14 levels increase after exhaustive exercise training [14]. Further studies are needed to better understand the mechanisms responsible for transcriptional upregulation and activation of the MMP family and to determine the biological significance of MMPs for the adaptation of the skeletal muscle to exercise.

It is currently unknown whether different exercise training programs, to include one that incorporate machine based resistance exercise and one composed of aerobic and body weight exercise such as pull-ups and sit-ups, promote different MMP

responses [95–97]. Such information would be valuable in understanding whether the type of exercise and the specific MMP response plays a role in mediating physiological adaptations to exercise training.

mRNA expression of heat shock proteins in muscle has been shown to occur depending on type and intensity of exercise [49]. Mahoney *et al.* [98] demonstrated that exercise differentially affected the expression of the genes involved in metabolism, cell growth and transcriptional activation as well as apoptosis. Genes that show identical regulatory patterns in leukocytes after exercise are (1) those associated with cell stress management e.g., HSP 90; (2) those associated with proteolysis e.g., MMPs; and (3) those associated with apoptosis e.g., Bcl-2 related anti-apoptotic proteins [11].

Although it is known that vigorous exercise and sex influences tendon metabolism and mechanical properties, it is unknown about the structural and regulatory components that contribute to the responses. It is also currently unknown how sex regulates mRNA expression of collagens, MMPs and TIMPs genes in tendons. Understanding the above will have important implications on why women have differential mRNA expression of MMPs and TIMPs than men.

Microarray technology in exercise physiology is an important tool being used currently for monitoring athletes training process. This is based on the intension of identifying exercise induced gene expression profiles or fingerprints that can be related to exercise intensity(ies) or type(s) [99]. Conceivably, an impact of exercise may be ascertained in the gene expression profiles in blood cells, for example, leukocytes and also exercising muscles and tendons.

The time course of increase in endostatin level may provide evidence of its possible vasoregulatory effect [85] i.e., endostatin could regulate blood supply during physical exercise in addition to its angiogenic effects [90]. MMPs such as MMP-2 and MMP-9 are also elevated after physical performance. Thus, intensified research on endostatin with respect to special physiological and mechanical stimuli that cause an increase in MMPs may lead to better understanding of vascular signaling during exercise in the prevention of a variety of diseases such as coronary heart diseases and diabetes.

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Regulation of Chondrocyte Matrix Metalloproteinase Gene Expression

Charles J. Malemud

Abstract Matrix metalloproteinases also known as MMPs are zinc-dependent endoproteases which process extracellular matrix proteins at neutral pH to regulate normal macromolecular protein turnover as well as those cellular events associated with the remodeling of tissue architecture. The MMP superfamily of proteins include the classical MMPs, MMP-1, -8, -13, and -18, the gelatinases, MMP-2, -9, stromelysins, MMP-3, -10, -11, matrilysins, MMP-7, MMP-26, membrane-type MMPs (MT-MMPs; MMP-14, -17, 24/25) the ADAMS (*a d*isintegrin *a*nd *m*etalloproteinase) also known as adamlysins and the ADAMTS (*a d*isintegrin *a*nd *m*etalloproteinase with *t*hrombo*s*pondin motif). MMP genes are regulated principally via the activation of transcription factors induced by various growth factors and cytokine/cytokine receptor interactions which are pertinent to the tissue allostasis, but which are most critical to the pathophysiological progression of rheumatoid arthritis and osteoarthritis. The most important transcription factors known to be involved in regulating MMP gene expression include the synthesis and/or activation of NF-κB, Cbfa1, AP-1, Nmp4/CIZ, ELF3, c-Maf, KLF5 and Sp1 which interact with specific known sequences in the promoter region of MMP genes. In arthritis, protein kinase pathways are generally activated by pro-inflammatory cytokines, including interleukin-1β, tumor necrosis factor-α, interleukin-6, and other members of the IL-6 protein family. The transcription factors activated by these signaling mechanisms have traditionally been considered "undruggable." However, recent experimental evidence indicates that inhibition of transcription factor synthesis and/or activation may be achieved which could be employed to suppress MMP gene activity.

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1 Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endoproteases [1, 2] which are considered essential for the maintenance of tissue allostasis. MMPs process extracellular matrix (ECM) proteins at neutral pH and in this way regulate normal ECM macromolecular protein turnover and those events associated the remodeling of tissue architecture [3].

Members of the MMP protein superfamily include the classical MMPs, MMP-1, -8, -13, and -18, the gelatinases, MMP-2, -9, stromelysins, MMP-3, -10, -11, matrilysins, MMP-7, MMP-26, membrane-type MMPs (MT-MMPs; MMP-14, -17, 24/25) [4], the ADAMS (*a d*isintegrin *a*nd *m*etalloproteinase) also known as adamlysins and the ADAMTS (*a d*isintegrin *a*nd *m*etalloproteinase with *t*hrombo*s*pondin motif [5, 6]. It is noteworthy that the transcriptional regulation of ADAMTS gene expression by chondrocytes in culture in response to pro-inflammatory cytokines has not been adequately studied, although Sylvester et al. [7] recently reported that the ADAMTS-4 (human aggrecanase-1) promoter contained an Sp1-binding site which was activated by treating cultured human chondrocytes with IL-1β. Furthermore, although the potential involvement of STAT-3 in regulating MMP-9 gene activity was reported in cultured human mammary epithelial cells [8], studies of this nature have not been systematically performed in chondrocytes.

This chapter focuses on the regulation of the MMP-1, -3, -9, -13 and ADAMTS-4 genes because of their importance as mediators of cartilage destruction in various type of arthritis (Table 1). These principal MMP types involved in arthritis are often modulated by a variety of growth factors and cytokine/cytokine receptor interactions which are also pertinent to the pathophysiological progression of RA and OA.

Table 1 Major MMP genes involved in RA and OA

MMP	Functions	References
MMP-1(interstitial collagenase)	Degradation—type I collagen	[10]
MMP-2 (72 kDa gelatinase)	Degradation—denatured Collagen; degradation—aggrecan	[5] [90]
MMP-3 (stromelysin-1)	Degradation of proteoglycans and other accessory matrix proteins	[5]
MMP-9 (92 kDa gelatinase)	Degradation—denatured collagen	[1, 11]
MMP-13 (collagenase-3)	Degradation—type II collagen, proteoglycans and other matrix proteins	[5]
MMP-14 (membrane-type MMP)	Activator of pro-MMP-2 and -13; degradation—aggrecan	[11, 91]
ADAMTS, -1, -4, -5 (aggrecanase)	Degradation—aggrecan, versican	[5, 92]

2 MMP Transcription and Synthesis

Most of the cytokine, hormone or growth factor-regulated MMPs are synthesized as latent enzymes. In general, conversion of the pro-enzyme to the active catalytic state is mediated by protein-activator proteins, which often includes plasminogen activator and the pro-hormone convertase, furin [9].

The activity of the activated MMPs is regulated by binding of endogenous tissue inhibitor of metalloproteinases (TIMPs) to the enzyme active site or to alternative TIMP-binding sites of an activated MMP [10]. Thus, up-regulation of MMP gene transcription, enzyme synthesis and activation when coupled to a reduced capacity of TIMPs to regulate activated MMPs was found to be strongly correlated and occurred in various pathological conditions associated with elevated levels of ECM protein degradation. Indeed such relationships have already been identified to occur in growth plate disorders, arthritis, cardiovascular disease, cancer, as well as ischemia, stroke and a few of the neurodegenerative disorders [9, 11–14].

This chapter will focus on the most pertinent molecular mechanisms known to be involved in the transcriptional regulation of MMP gene expression as well as the signal transduction pathways [4, 15–19] activated by pro-inflammatory cytokines, including interleukin-1β (IL-1β) tumor necrosis factor-α (TNF-α), IL-6, the IL-6 family member, adiponectin and other hormones, such as parathyroid hormone. We will also critically analyze the extent to which transcription factors (heretofore considered "undruggable") which can be activated via stress-activated protein kinases/mitogen activated protein kinases (SAPK/MAPK), Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) and cyclic AMP (cAMP) signaling pathways and are known to participate in the regulation of MMP gene transcription, may be modifiable or their activity inhibited, thus leading to a dampening of MMP gene activity. Finally, because increased levels of specific MMPs in arthritic synovial fluid and peripheral blood, such as MMP-1, MMP-3, MMP-9 and MMP-13 are so critical to the breakdown of articular cartilage ECM proteins [20–23], a discussion of the cellular mechanisms underlying pro-inflammatory cytokine regulation of MMP gene transcription in chondrocyte cultures initiated from osteoarthritic (OA) and rheumatoid arthritic (RA) human cartilage is also warranted.

3 Transcription of MMP Genes

The results of numerous studies that proved that the synthesis of MMPs was primarily regulated by transcription are now almost two decades old and this research area was comprehensively summarized by Westermarck and Kähäri [24]. During the period when many of these studies occurred, it was also conjectured that both transcription *and* post-translational mechanisms, including alterations in MMP mRNA stability [25, 26] and more recently, epigenetic mechanisms [27], could also account for the modulation of MMP gene activity.

Table 2 Transcription factors that regulate MMP gene expression

Transcription factor	MMP regulation	References
NF-κB	MMP-1; MMP-13	[36, 93]
Cbfa1	MMP-13	[40, 42]
AP-1	MMP-13	[44, 46]
Nmp4/CIZ	MMP-13	[48]
ELF3	MMP-9; MMP-13	[52, 53]
c-Maf	MMP-13	[61]
KLF5	MMP-9	[63]
Sp-1	MMP-9	[94]
	ADAMTS-4	[7]
STAT-3c	MMP-9	[8]

Certain MMP gene activities are constitutively synthesized by many cell types. However, overall, the observed changes in ECM protein structure during connective tissue remodeling was shown to result from the activity of inducible promoters regulated by various mitogens and/or growth factors, cytokines, endotoxin, hormones, stress and cellular transformation [2]. Thus, the biological activity of these factors at the cellular level leads to either positive or negative regulation of MMP genes under the control of various transcription factors via their interaction with regulatory elements in human MMP gene promoter regions (Table 2).

4 Transcription Factors

4.1 Nuclear Factor-κB (NF-κB)

The NF-κB signaling cascade represents one of the most critical components involved in the inflammatory response [28]. Activation of NF-κB by pro-inflammatory cytokines [29] is one of the foremost mechanisms in RA that can cause up-regulation of MMP gene activity [30–32], the destruction of articular cartilage, as well as resistance of synovial tissue to induction of apoptosis [33–35]. With respect to NF-κB activation and MMP gene activity, activated NF-κB components, p50/p52/p65 form homo/heterodimers in the nucleus where they bind to cognate enhancer sequences in the promoter region of MMP-1, MMP-3, MMP-9 and MMP-13 genes. This mechanism is generally considered to be relevant to the up-regulation of expression of these MMP genes. However, the results of studies by Kim et al. [36] showed that inhibition of NF-κB activity by pyrrolidine dithiocarbamate (as well as other NF-κB inhibitors) up-regulated, rather than down-regulated MMP-1 and MMP-13 gene activity in RA synovial fibroblast cultures stimulated with IL-1β. These results suggested that changes in MMP gene activity thought to occur solely on the basis of NF-κB activity could also be regulated by NF-κB-independent mechanisms (see below).

4.2 Core Binding Factor-α1/α2 (Cbfa1) and Activator Protein-1 (AP-1)

Cbfa1 belonging to the *runt* gene family (i.e. Runx2) was implicated in regulating the rate of bone matrix deposition by differentiating osteoblasts [37] and in MMP-13 gene expression [38, 39]. Indeed, the MMP-13 promoter region was shown to possess a TATA box enhancer element, a Cbfa1site, an activator protein-1 (AP-1) site, a polyomer enhancer A binding protein-3 (PEA-3) site, and a TGF-β inhibitory element (TIE). Based on the ability to identify these structural elements in the MMP-13 promoter, Selvamurugan et al. [40] used the rat osteoblastic cell line, UMR-106-01, to show that TGF-β_1 stimulated the transcription of the MMP-13 gene which was characterized by the transactivation of the Cbfa1/Mothers against decapentaplegic homolog-2 (Smad-2) regulatory site.

The AP-1 site and the runt-domain-binding (Runx/RD/Cbfa) site as well as their respective binding protein partners, C-Fos/C-Jun and Cbfa1 were also shown to be critical for regulating rat MMP-13 promoter activity in parathyroid hormone (PTH)-treated and differentiated osteoblast cultures [41]. More recently, in addition to AP-1 as a site for regulating MMP-13 gene activity, Schmucker et al. [42] identified a conserved region located 20 kb upstream from the MMP-13 transcription start site. This conserved region included a distal transcription response element for MMP-13 which was shown to contribute to MMP-13 gene expression in chondrocytes treated with IL-1β.

4.2.1 Signal Transduction and AP-1

A critical study focused on linking activation of the SAPK/MAPK signaling pathway to the up- or down-regulation of MMP-13 gene expression. In that regard, small molecule inhibitors (SMIs) of extracellular-regulated kinase 1/2 (ERK 1/2) and p38 kinase, but not C-Jun-NH_2-kinase (JNK), suppressed MMP-13 gene activity through inhibition of binding of phosphorylated-ERK 1/2 (P-ERK 1/2 and p-38 kinase) to the AP-1 site [43]. In a companion analysis, basic fibroblast growth (bFGF), a critical growth factor in the progression of RA [44, 45] was shown to stimulate MMP-13 gene expression via occupancy of the AP-1 site by members of the JUN family which include c-Jun, JunB, JunD, c-Fos, FosB and Fra2 [43].

Most recently, Lim and Kim [46] showed that only the p38 inhibitor, SB203580, blocked MMP-13 induction and AP-1 activation in IL-1β-treated SW1353 chondrocytes. SB203580 also inhibited the nuclear translocation of c-Fos, but not the translocation of c-Jun. Of note, treatment of SW1353 chondrocytes with IL-1β also induced activation of Janus kinase 2 (JAK2) and signal transducer and activator of transcription-1 and -2 (STAT-1, -2) activation. Thus, the JAK2 inhibitor AG-490 blocked STAT-1, STAT-2 activation as well as the induction of MMP-13 gene transcription in IL-1β-treated SW1353 chondrocytes. In further support of this result, transfection of SW1353 chondrocytes with STAT-1, -2 silencing RNA siRNA also reduced

MMP-13 expression levels. The results of these studies pointed out the complexity of regulating chondrocyte MMP-13 gene expression in response to treatment with IL-1β or bFGF as occurring through activation of SAPK/MAPK signaling, JAK/STAT signaling as well as the appropriate binding of activated Cbfa1 and members of the JUN protein family to the AP-1 site in the MMP-13 promoter. Both AP-1 and Cbfa1 were required for induction of MMP-1 (collagenase-1) by low concentrations of PTH employed at 10^{-8} M rat PTH-fragment (1–34) in cultured osteoblast-like cell lines, ROS 17/2 and UMR 106 [47]. Importantly, the introduction of mutations in the CCACA binding site for Cbfa1 eliminated induction of MMP-13 and PTH-mediated transactivation of Cbfa1/Runx proteins. In addition to Cbfa1, a minor complex was also observed which may have been due to binding of the Cbfa2 protein.

4.3 Nucleocytoplasmic Shuttling Transcription Factor-4 (Nmp4)/Cas Interacting Zinc Finger Protein (CIZ)

Nmp4/CIZ was shown to regulate osteoblast MMP-13 promoter activity in response to PTH [48]. In order to better understand the relationship between Nmp4/CIZ and MMP-13 promoter activity, Alvarez et al. [49] characterized the 5′ regulatory region of the mouse Nmp4 gene, located on chromosome 6. The results of these studies showed that two adjacent promoter regions called P(1) (−2521 nucleotide/−597 nucleotide) and P(2) (−2521 nucleotide/+1 nucleotide) initiated transcription of alternative first exons U(1) and U(2). Both of these promoter regions lacked TATA and CCAAT boxes. However, these promoters did contain initiator sites and CpG islands. The promoter regions were active in both osteoblast-like cells and in the M12 B-lymphocyte cell line and of note, low concentrations of PTH attenuated P(1)/P(2) activity. The Nmp4/CIZ promoter activity was shown to undergo autoregulation with deletion analysis identifying regions that could drive P(1) and P(2) basal activities as well as other regions that contained both positive and negative regulatory elements affecting transcription.

The Nmp4/CIZ proteins have also been implicated in fluid shear stress (FSS)-stretched MC3T3-E1 osteoblast-like cell MMP-13 transcription [50]. Thus, FSS increased MMP-13 gene transcription in these cells as a result of decreased Nmp4/CIZ binding to its cis-element within the PTH response region. In support of this result, a mutation introduced into the PTH response domain abrogated the MMP-13 response to FSS.

4.4 E74-Like Factor 3 (ELF3)/Ets Domain Transcription Factor

The conserved E26 transformation-specific sequence (ETS domain-related transcription factor, ELF3) has a conserved Ets domain that belongs to the winged

helix-turn-helix DNA-binding domain superfamily. ELF3 has been implicated in the progression of various forms of cancer, such as, Ewing sarcoma, acute myeloid leukemia and chronic myelomonocytic leukemia [51].

ETS binding sites, including the ELF3 and ESE-1 sites, were found in the promoters of the MMP-13 and MMP-9 genes [52, 53] where the ELF3 binding site is located proximal to AP-1 and Runx2. Recently, ELF3 binding to the MMP-13 promoter was shown to be enhanced by treatment of human chondrocytes with IL-1β [54]. Moreover, MMP-13 gene expression was suppressed by siRNA-ELF3. Of note, activation of ERK 1/2 by treatment with IL-1β enhanced ELF3-stimulated MMP-13 promoter activity with higher levels of ELF3 co-localizing with MMP-13 protein in human OA cartilage.

4.5 c-Maf

The transcription factor, Maf, also known as protooncogene c-Maf or v-Maf musculoaponeurotic fibrosarcoma oncogene homolog is encoded by the *MAF* gene [55]. Maf has been reported to interact with the cyclic AMP response-element binding protein and EP300 through the MAF recognition element [56] and with Myb [57]. However, c-Maf may have special significance for understanding the biology of chondrocytes because the long form of c-Maf (Lc-Maf) was shown to cooperate with SOX9, a cartilage-specific transcription factor [58], to activate the Type II collagen gene [59].

Maf and the neural retina specific gene, Nrl can bind to the AP-1 site where they form heterodimers with Fos and Jun [60] making it likely that c-Maf would be involved in regulating MMP gene activity. Indeed, Li et al. [61] showed that c-Maf significantly increased MMP-13 promoter activity in chondrocytes derived from OA cartilage. Moreover, chromatin immunoprecipitation analysis showed that c-Maf bound to the MMP-13 promoter in a region containing the AP-1 site.

4.6 Krüppel-Like Factor 5 (KLF5)

KLF5 is also known as intestinal enriched Krüppel-like factor (IKLF) or basic transcription element binding protein 2. KLF5 was shown to bind to p300 which causes acetylation of the first zinc finger and confers a transactivating function on KLF5 [62].

KLF5 was shown to be expressed by chondrocytes and osteoblasts, but not by osteoclasts [63]. In this study, cartilage growth and differentiation occurred normally in KLF5$^{+/-}$ mice. However, ECM protein degradation was abnormal and growth plate development characterized by a delay in endochondral ossification. Of note, over-expression of KLF5 in the chondrogenic cell line, OUMS27, resulted in the degradation of gelatin by stimulating the promoter for MMP-9. These results indicated that KLF5 was a transcriptional regulator of MMP-9 gene activity and was therefore likely to be a critical modulator of MMP-9 in developing long bones [11].

5 Modulation of Transcription Factor Activity: Does Inhibiting Transcription Factors Suppress MMP Gene Activity?

In RA, partial suppression of MMP synthesis increased by the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α has generally been achieved through the development of disease-modifying anti-rheumatic biological drugs including, an IL-1 receptor antagonist, an anti-IL-6 receptor antagonist and several anti-TNF-α antagonists. These biological agents neutralize the interaction between these pro-inflammatory cytokines and their respective cognate receptors. Additionally, a novel small molecule inhibitor, tofacitinib, was shown to inhibit the JAK/STAT downstream signaling pathway which is activated by IL-6, and by other growth factors and hormones [64]. Of note, a recently published novel multibiomarker disease activity score for use in determining the progression of RA disease activity [65] included two pro-inflammatory molecules pertinent to the up-regulation of MMP gene activity, namely, IL-6 and TNFR1 [66] and two MMPs, namely, MMP-1 and -3.

Modulating transcriptional factor activity with the ultimate goal of suppressing inflammation has been a research focus over the past decade or so. It has been also been thought that by reducing the interaction of various transcription factors known to be active in regulating MMP gene promoter activity, inhibition of MMP gene transcription could also be achieved as well.

5.1 NF-κB

Because NF-κB is considered the 'master' mediator of inflammatory responses, targeting NF-κB has long been sought after as a strategy to suppress inflammation [67–69]. Indeed, several hundred inhibitors of NF-κB activity, including peptides and polypeptides, anti-oxidants, small RNA/DNA molecules, and dominant-negative NF-κB constructs have been studied mostly towards the goal of inhibiting induction of activated NF-κB [68]. However, most recently, a novel strategy employing "programmable DNA minor groove binders" were shown capable of binding to the κB sites of the inhibitory NF-κB complex [70]. Employing this strategy reduced the expression of IL-6 and IL-8 driven by activated NF-κB to a level that overlapped with the use of the NF-κB inhibitor, PS1145. Thus, inhibiting NF-κB/DNA binding by direct displacement may ultimately prove to be a useful alternative to employing existing chemical or peptide antagonists of NF-κB activity. Of note, the extent to which this strategy will also modulate the expression of MMP genes has not yet been determined.

5.2 Cbfa1

Significant progress has been achieved using various experimental strategies developed to impede the activity of Cbfa1 but there has been little in the way of advances

in employing these techniques to inhibit MMP gene activity. One strategy has been to suppress the expression of CBfa1. In one study, a prototypical osteoblastic cell line, UAMS-33 was shown to express the Cbfa1-a/Osf2 transcription factor, but not peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) [71], the latter being a required transcription factor for adipocyte differentiation. Stable transfection of UAMS-33 with PPAR-γ2 and activation of PPAR-γ2 with the thiazolidineodione, BRL 49653 resulted in changing these cells to an adipocyte phenotype which was accompanied by suppression of Cbfa-1/Osf2 gene expression, as well as inhibition of α (I) procollagen, osteocalcin synthesis and mineralized matrix formation. These results indicated that those cells expressing PPAR-γ2 under these conditions could result in reduced expression of Cbfa-1/Osf2. Suppression of Cbfa1 synthesis was achieved by stimulating the accumulation of intracellular cAMP with the forskolin in the osteoblastic cell line, MC3T3-E1 [72], by activating osteoblast Smad3 with TGF-β [73] or by treatment of MC3T3-E1 cells with TNF-α [74]. Production of the Cbfa1 isoform and osterix mRNA was inhibited by FK506 in ROS 17/2.8 cells [75]. Additional strategies employed to inhibit the activity of Cbfa1 have included, adenoviral-mediated transfer of siRNA against Cbfa1/Runx2 *in vitro* and *in vivo* where an siRNA against Cbfa1/Runx2 inhibited heterotopic ossification induced by bone morphogenetic protein-4, demineralized bone matrix and trauma [76] and by the application of Id proteins which play an important role in osteogenic differentiation [77]. In the latter study, p204, a member of the Id protein family, bound to Cbfa1 blocked the transcription of alkaline phosphatase and osteocalcin. Thus, one can postulate that some of these experimental strategies may be amenable to study the extent to which blockade of Cbfa1 gene expression or Cbfa1 binding to MMP promoter regions in the presence and absence of relevant pro-inflammatory cytokines will inhibit MMP gene transcription.

5.3 AP-1

The critical role of AP-1 as a regulator of MMP gene activity has produced a number of experimental studies targeting several of the adaptor proteins which regulate AP-1 gene activity, AP-1 activity directly or the signaling pathways that lead to AP-1 activation, all with the ultimate goal of down-regulating MMP gene transcription. In the first example, Ahmad et al. [78] showed that RNA interference which reduced the expression of the adaptor proteins, myeloid differentiation primary response gene-88 (MyD88), interleukin-1 receptor-associated kinase 1 (IRAK1) or the E3 ubiquitin ligase TRAF6, also caused inhibition of MMP-13 promoter activity in human chondrocyte cultures treated with IL-1β. Moreover, the lower level of MMP-13 gene expression was found to be dependent on the activation of ERK 1/2, p38 kinase, JNK and c-Jun as well as AP-1 binding activity.

Because the activation of the SAPK/MAPK pathway by pro-inflammatory cytokines has been shown to result in an increase in MMP gene activity in RA [79–81], inhibition of this signaling pathway with specific small molecule inhibitors has also

been tested as a technique to down-regulate, NF-κB, AP-1 and MMP gene activity [82–85]. The results of three studies, in particular, are also noteworthy because they showed that dampening of AP-1 activity suppressed MMP production.

Hui et al. [86] showed that the mitotic inhibitor, paclitaxel, employed in cancer chemotherapy, reduced AP-1 activity and AP-1/DNA binding to chondrocytes treated with IL-1β. MMP-1 and MMP-3 activity was also reduced. Importantly, paclitaxel did not alter the expression of c-Fos or c-Jun mRNA nor did paclitaxel affect chondrocyte viability or the expression of aggrecan or Type II collagen.

The polyphenolic fraction of green tea extract, epigallocatechin-3-gallate (EGCG) [87] was also shown to inhibit AP-1 and NF-κB activity in human chondrocyte cultures. The EGCG effect correlated with the reduced gene expression of MMP-1 and MMP-13 [88].

Finally, Boileau et al. [89] employed licofelone, a dual cyclooxygenase (COX) and 5-lipoxygenase inhibitor (5-LOX), the COX-2 specific experimental inhibitor, NS-398 or BayX-1005 (a 5-LOX inhibitor) to test the hypothesis that inhibition of PGE_2 and 5-LOX were both required to dampen MMP-13 gene expression by chondrocytes derived from human OA cartilage. Licofelone was shown to inhibit MMP-13 gene expression, but neither NS-398 nor BayX-1005 had this effect indicating that MMP-13 gene expression was likely to be dependent on both PGE_2 and 5-LOX synthesis. Of note, licofelone had no effect on the activation of ERK 1/2 or JNK but did decrease the activation of c-Jun, p38 kinase, CREB and AP-1.

The results of these experimental studies all predicted that manipulating SAPK/MAPK signaling and AP-1 activity, and for that matter NF-κB, would result in reduced MMP gene activity in RA and OA. However, little progress has been made on translating the results of these pre-clinical studies into the development of novel therapeutic treatments for arthritis which would be based on targeting these molecules.

6 Conclusions

MMP gene activity is controlled in cultured articular chondrocytes, osteoblasts and synoviocytes primarily at the level of transcription. Synthesis and activation of diverse transcription factors, including, NF-κB, Cbfa1, AP-1, Nmp4/CIZ, ELF3, c-Maf, KLF5 and Sp1 together with their capacity to interact with specific sequence motifs in the promoter region of MMP genes modulates MMP synthesis. In cultured synovial cells, osteoblasts and chondrocytes from RA and OA synovial joints, the pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β which are found at elevated levels in the synovial fluid of RA and OA joints are potent stimulators of MMP gene activity via the activation of these transcription factors. At present, strategies designed to modulate MMP gene activity in RA and OA have focused mainly on neutralizing cytokine/cytokine receptor interactions or on inhibiting protein kinases that regulate downstream cellular events pertinent to immune function and inflammation. Although traditionally, transcription factors have been long been considered as “undruggable” targets, persuasive evidence exists from pre-clinical studies to now consider

inhibition of transcription factor synthesis and/or transcription factor activity as alternative approaches to modulating MMP gene activity in the arthritides.

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Microbial Source of Fibrinolytic Protease and Its Biotechnological Potential

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Abstract Many fibrinolytic proteases are isolated and purified from various sources. These fibrinolytic proteases have biotechnological applications to treat cardiovascular diseases. Fibrinolytic enzyme, Actinokinase is isolated and produced from thermophilic *Streptomyces megasporus SD5*. The fibrinolytic enzyme is serine endopeptidase. The proteolytic enzyme which acts on different substrates casein, azocasein, fibrin etc. Enzyme is plasminogen independent. Some of these properties are studied & summarized for isolated fibrinolytic protease. Various fibrinolytic proteases also isolated from different resources and their biochemical characterization is reported in the literatures. In general proteases are also used in food, pharmaceutical, cosmetics and environmental industry. Overall all proteases have been used for application prospectives. There are many fibrinolytic proteases discovered till todate. These agents need to check thoroughly to their biochemical properties and substrate base catalysis. Therefore microbial fibrinolytic proteases are used for therapeutic approach of medical interest. The diverse microbial population produces the fibrinolytic proteases. The properties of fibrinolytic enzymes are described in different literatures. Many reported fibrinolytic enzymes are plasminogen activator and some are fibrin specific in nature. These enzymes had drawbacks such as bleeding complications, haemorragic in nature and expensive in nature. So search of new fibrinolytic proteases is still in continued for better molecule.

Keywords Microbial enzymes • Fibrinolytic protease • Thrombolytic • Actinokinase

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1 Introduction

Proteases are important enzymes which contribute significant part of the world enzyme production [1]. Microbial cells which were isolated from various places in environment and reported for their biotechnological potential for producers of proteases, also produce different types of proteases which are used for various industrial applications. They are useful in food, pharmaceutical and environmental industry [2].

Some proteases act on different substrates and convert into the products which are useful for food and fodder applications. The Chitte *et al*. [3] studies on partial characterization of keratinase enzyme acts on different substrates and releases the useful amino acids. These types of proteases are useful in poultry feather removal and could be useful for application point of view.

Microbial sp. have been studied for their enzyme biocatalytic potential in various fields. Now enzyme base diagnostic kits are also useful in various commercial uses. These are used in fermentation as a sensor as well as in diagnostic tests. e.g. the creatine kinase is used in diagnostic for rapid detection in heart attack.

Streptomyces sp. were isolated from western coast of Maharashtra which secrete useful multiple enzymes. These are Fibrinolytic, amylase, keratinase, lipase, cellulose among this fibrinolytic enzyme is studied in detail.

The thermophilic *Streptomyces sp*. is isolated from hot spring water of Western coast of Maharashtra. These species produce the multiple enzymes which have applications in various fields. These enzymes are fibrinolytic, cellulase, endonucleases, lipase etc. The topoisomerases like endonucleases have been isolated and partially characterized from thermophilic microorganisms. The results of these types of enzymes are unpublished. Microbial isolates from soil screened qualitatively for L-glutaminase and L-Asperginase enzyme and further characterization is in progress.

Microbial enzymes have tremendous biotechnological, biomedical and pharmaceutical applications. Protease constitute major commercially available enzymes [4]. Microorganims are valuable sources of proteases due to their short generation time and easy large scale production and recombinant DNA technology used in various sectors [5]. Enzyme engineering aspect is also used for improving the activity and stability of enzyme.

Among these alkaline proteases have huge biotechnological potential for industrial sectors like laundry detergents, paper and pulp, leather processing, brewing, food and pharmaceutical industries [6].

Thermophilic actinomycetes are producers of thermostable enzymes. These enzymes exhibit several advantages due to high processing temperature which leads to increased reaction rates, solubility and contamination chances are less. Beside to this they have higher stability towards other protein denaturating conditions.

Extreme environments are proved to be good sources of microbial population. The isolates are used for production of industrially important micromolecules. The micromolecules can be used in various sectors like food, detergent, pharmaceutical industries. In pharma drug development process is long run and need patience with respective to financial sources and it's outcome.

In MACS Collection of Microorganisms (MCM, affiliated to WFCC, WDCM code no. 561), is an in-house culture collection of different microbial species. Among these *Streptomyces sp*. is one of them, well studied for the production and characterization of fibrinolytic protease its name is 'Actinokinase.' Currently available enzymes have been reported for their drawbacks like bleeding complications, immunogenic and expensive etc. So search of new and better fibrinolytic protease is continuing from various sources. Fibrinolytic enzymes are given attentions for their potential to use as clot dissolving agent. There are several fibrinolytic enzymes characterized and reported from various sources. So search of new fibrinolytic proteases is in continued for potent, safe and economical molecule.

2 Microbial Sources of Fibrinolytic Protease

The enzyme uses or microorganisms as biocatalysts for various applied applications have been reported in the literatures [7]. Some of the industrial process conditions are harsh. There are needs for enzymes that can withstand the process conditions. The most of the enzymes are reported from mesophilic microorganisms and advantages of using these enzymes is restricted due to their stability at extremes of temperature, pH and salt concentrations. Extremophiles specifically thermophiles are a potent source of biomolecules like fibrinolytic protease, antibiotics, lipase, cellulase and endonuclease type of topoisomerase. These biomolecules are tested from thermophilic *Streptomyces sp*. Among these fibrinolytic protease is described and properties are compared with other reported fibrinolytic enzyme from reported microbial sources.

Microorganisms are potential sources of therapeutic enzymes. Various microbial species produce enzymes which have diverse active site nature for its enzyme activity.

Many microbial enzymes have been isolated and characterized. The microbes which are isolated from different ecosystem are studied by different research groups. Some of them used native or wild gene constructed and expressed for quality improvement of desired potential molecules. e.g. construction of different recombinant molecules for the production of value added products such as proteases used in food industry, pharmaceuticals like fibrinolytic enzymes. The sources of microbial fibrinolytic enzyme is listed in Table 1. Fibrinolytic protease was purified from *Streptomyces sp*. CS684 with the aim of isolating economically feasible enzyme from microbial source. This enzyme is highly similar to serine protease, the result suggest that FP84 is a novel serine metalloprotease with potential application in thrombolytic therapy [22]. Microorganisms are potential sources of therapeutic enzymes for applied uses these enzyme for therapeutic purposes is limited because of their incompatibility with the human body.

Fibrinolytic alkaline proteases like *subtilisin E*, *BPN* etc. have highly homologous sequence with the corresponding fibrinolytic enzymes so what is the reason behind that only fibrinolytic enzymes have very high substrate specificity to fibrin? It might be evolutionary change of the critical amino acid residues in the substrate binding site probably reason for this difference [23].

Table 1 Sources of microbial fibrinolytic enzymes

Source of microorganism	References
Streptomyces megasporous SD5	[8–12]
Streptomyces rimosus	[13]
S. Violaceoruber	[14]
S. Spiroverticillatus	
B. subtilis LD 8547	[15]
Bacillus subtilis LSSE-22	[16]
Bacillus subtilis LD-8547	[17]
B. subtilis A1	[18]
B. subtilis 168	[19]
Pseudomonas sp. TKU015	[20]
Bacillus subtilis TKU007	[21]

3 Characterization of Actinokinase: A Fibrinolytic Protease

The *Streptomyces sp*. isolated from Western coast of Maharashtra and identification as well as isolation of enzyme is carried out. The fibrinolytic protease enzyme 'actinokinase' is isolated purified and characterized. Its biotechnological potential in medical field is possible. The characterization of fibrinolytic type of proteases is summarized in Table 2 [24]. The fibrinolytic enzyme showed the activity using substrates casein, azocasein, fibrin, using fibrin plate assay as qualitative detection of fibrinolytic activity of isolated enzyme. The enzyme designated as name 'Actinokinase' has E.C. # 3.4.21.23. The enzyme is serine endopeptidase classified as hydrolase type of enzyme which breaks the fibrin filaments and dissolves the clot.

The fibrinolytic protease 'actinokinase' is serine endopeptidase and its properties and comparison of characteristics with other fibrinolytic proteases are briefly given in Tables 2 and 3. The fibrinolytic protease 'Actinokinase' is plasminogen independent which acts directly on fibrin and subsequently lyzed the fibrin clot. The enzyme spectrum is closely similar to commercial available Urokinase enzyme spectrum (Fig. 1). The partial sequence of the enzyme also used for prediction of phosphorylation sites on amino acids using NetPhos 2.0 Server. In that 'Threonine' 477 residue has possibility of phosphorylation during the substrate catalysis (Fig. 2).

The listed reported fibrinolytic enzymes classified as per their characterization and listed in the comments section of Table 4. These fibrinolytic proteases are isolated from various sources of microorganisms and described as in literatures. The comparison of the characteristics of enzyme 'actinokinase' is given with other sources of fibrinolytic proteases.

The partial gene sequence also deposited its accession # AY289795.1. The enzyme encoding gene (Act S) partial genomic libraries were prepared. Cloning and expression of thermophilic *Streptomyces* 'actinokinase' gene also described [25]. The fibrinolytic enzyme 'actinokinase' is a Chymotrypsin type of protease and its active site contains 'Serine,' His and Asp like serine peptidase a catalytic triad present in the active site.

Table 2 Properties of fibrinolytic enzyme 'Actinokinase'

Properties	Fibrinolytic protease 'Actinokinase'
Class	Serine peptidase
Clan	SA
Family	S1
Molecular weight	~35 kDa
pI	7.56
pH stability	5–9
Plasminogen independent	Independent

Table 3 Comparison of the characteristics of fibrinolytic enzyme 'actinokinase' with other fibrinolytic serine protease

Source of microorganisms	Molecular weight (kDa)	Production of enzyme at optimum temperature	Enzyme activity at optimum temperature	Comments	References
S. megaspores SD5	29–35 kDa Actinokinase	55 °C for 18 h	37 °C	Chymotrypsin like serine peptidase	[12]
Bacillus sp. Nov. SK006	43–46 kDa	37 °C for 36 h	30 °C	Serine protease	[26]
Streptomyces sp. CS684	35 kDa FP84	28 °C for 48 h	45 °C	Serine metalloprotease	[22]
Bacillus subtilisin QK-2	QK-1—42 kDa QK-2—28 kDa	37 °C for 24–48 h	55 °C 37 °C	Subtilisin-family serine protease	[27]
Pseudomonas sp. TKU015	Nattokinase 21 and 24 kDa	37 °C for 2 days	37 °C	Serine protease	[20]

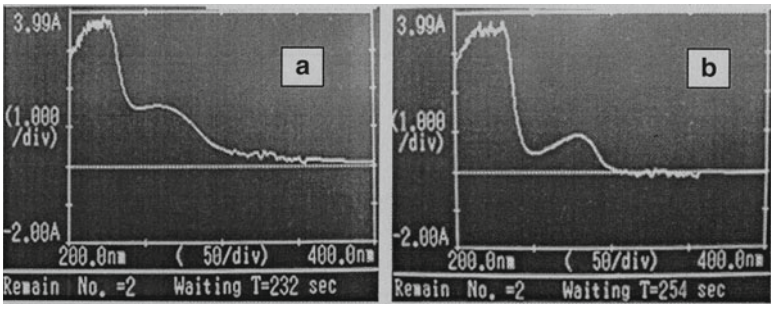

Fig. 1 Spectra scan: (**a**) Actinokinase enzyme; (**b**) Urokinase

Various microbial fibrinolytic proteases are isolated purified and characterized [23]. These enzymes are 'fibrinolytic' but they are dissimilar with each other with some properties. Some are similar with molecular weight but different biochemical properties. Some may have same catalytic triad but might have different specificity with respective to substrates. 'Streptokinase' is plasminogen activator that generates plasmin (proteolytic enzyme) which finally cleaves fibrin so streptokinase exhibited indirect specificity. While so designing of the recombinant and chimeric forms have direct specificity towards fibrin.

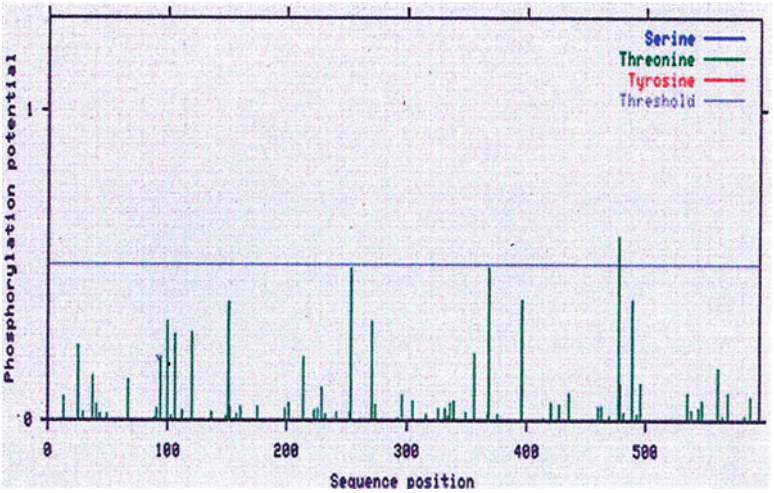

Fig. 2 Net 2.0 predicted phosphorylation sites in partial sequence of fibrinolytic protease 'actinokinase'

Table 4 Comparison of the characteristics of fibrinolytic enzyme "actinokinase" with other fibrinolytic serine protease

Source of microorganisms	Molecular weight (kDa)	Production of enzyme at optimum temperature	Enzyme activity at optimum temperature	Comments	References
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4 Production of the Fibrinolytic Protease

The medium optimization is generally critical for the fermentation production of fibrinolytic enzymes. Different microorganisms posses diverse physiological characteristic. So it is essential to optimize media components and environmental parameters for cell growth and fibrinolytic production [28]. The optimum temperature condition for *S. megasporus SD5* for enzyme production is 55 °C as the microorganism was isolated from a hot spring [9].

The fibrin enhances the fibrinolytic protease production that mean fibrin act as activator or inducer [29]. The nitrogen source peptone and yeast extract seemed to be effective for production of fibrinolytic protease. Potassium nitrate, elastin, methionine and phenylamine might not be suitable substrates for *S. megasporus strain SD5* could produce only 36–45 % enzyme production. The trace metal solution and phosphate enhanced the production of different proteases but these could not increase the production of the thrombolytic enzyme produced by *S. megasporus SD5*.

The growth curve for production of fibrinolytic protease 'actinokinase' is studied. The enzyme production is carried out at 2.5 L as well at 10 L fermentor. The cell free broth containing enzyme is passed through the ion exchange column which is equilibrated with 10 mM phosphate buffer and eluated with range of 0.5–1.0 M sodium chloride containing buffer. The fractions are collected and *ex-vivo* enzyme activity is carried out. The protein content and assay of clot lysis is performed. *S. megasporus* produces ~150–200 mg/L of crude enzyme. Activity is estimated about ~20,000 U/mg of pure enzyme.

In general thermophilic proteases or fibrinolytic production media and extreme temperature and pH for alkalophilic microorganism is required. These conditions are incompatible with standard industrial fermentation and downstream processing unit. Fermentation at high temperature is required. Specially designed equipment with cooling circulation is necessary for fermentation. Much work is still needed for developing methods by which thermophiles can be effectively cultivated for increased production of enzymes and biomolecules. e.g. To improve biomass production, different research groups developed different techniques such as optimization of medium composition [30] etc. most of the enzyme express and produce using recombinant DNA technology.

5 Prospectives of Fibrinolytic Protease

Researchers have screened and isolated microorganisms for enzyme production with high fibrinolytic activity. Most of the commercially available enzymes have drawbacks such as breeding complications, allergenic in nature, secondary immune response and expensive etc. There is need to search better and safe fibrinolytic protease which can be used to treat cardiovascular diseases.

There are many fibrinolytic enzymes reported from different sources. In this manuscript highlighted the only microbial sources. These enzymes are needed to investigate thoroughly for their respective uses.

One of the fibrinolytic protease name 'Actinokinase' is characterized and purified for the further studies [12]. The *ex-vivo* clot lysis also showed the fibrinolytic enzyme is potent in nature and further studies are needed. Investigation also showed that the enzyme is non-toxic in nature and also producer microorganism is non-pathogenic in nature.

'Nattokinase' is reported and isolated from different sources. The enzyme is serine protease currently used as oral drug. The action of the enzyme is directly on fibrin as well as it activates plasminogen [21].

Likewise actinokinase also acts directly on fibrin and is non-hemolytic in nature as this enzyme is extracellular in nature, short production time reduced the cost of production.

Thermoalkaliphilic enzymes have great biocatalytic potential in processes that are performed at alkaline pH and high temperature e.g. protease, lipase and cellulases are used as additives in laundry and dishwashing detergents [7]. Proteases are also used for dehairing of hides and skin to improve smoothness and dye affinity of wool [31]. For therapeutical point of view enzyme should be compatible to human body. So this fibrinolytic protease 'actinokinase' is chymotrypsin group. Microbial proteases have various applications in food, pharmaceutical and environmental industry. The diverse isolated microbial population from various sources produces unique enzyme which can exploit for their uses in respective field. Microbial enzymes are being studied from different microorganisms in various ecosystems. The different ecosystems adoptions under stress conditions produce micromolecules by extremophiles

6 Conclusion

In general enzymes industries have been huge market in the world. Health care and pharmaceutical industry is being recognized as an important request for commercial uses of enzymes. Enzymes have been requirements for uses as therapeutic agents against many diseases. The proteolytic enzymes which are isolated and characterized have biocatalytic potential as per report in the literatures. There is scope to isolate unique enzymes from different resources for their respective catalytic potential. Specially fibrinolytic proteases described for isolation, characterization and their uses need to explore. Microbial resources as a fibrinolytic protease can be useful to treat cardiovascular diseases. In this manuscript the author has addressed the microbial source as a fibrinolytic protease 'actinokinase' and listed with other fibrinolytic proteases as per literature report.

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Aspartic Proteolytic Inhibitors Induce Cellular and Biochemical Alterations in Fungal Cells

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Abstract Although fungal infections contribute substantially to human morbidity and mortality, the impact of these diseases on human health is not widely appreciated. Diagnosis and treatment of fungal infections remain a challenge in medicine despite recent major advances. The search for novel pharmacological compounds with antifungal action is a real requirement. Taking it into consideration, research groups have investigated the effects of aspartic peptidase inhibitors (PIs) on the development of human fungal pathogens such as *Candida* spp, mainly *Candida*

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albicans and *Candida parapsilosis*, *Cryptococcus neoformans*, *Pneumocystis jiroveci* and *Fonsecaea pedrosoi*, based on the following premises: (1) this class of hydrolytic enzymes performs multiple relevant roles in pathophysiological events associated to the fungal infections and (2) the introduction of human immunodeficiency virus (HIV) PIs in the clinical arena drastically reduced the opportunistic infections caused by fungi in this population. As expected, the blockage of one of these physiological/pathological processes should help in containing the fungal infection. Corroborating this hypothesis, both *in vitro* and *in vivo* studies have reported that classical aspartic PIs (e.g., pepstatin A) as well as HIV PIs (e.g., nelfinavir, saquinavir, ritonavir, indinavir, amprenavir, lopinavir and tipranavir) have induced several cellular and biochemical alterations on fungal cells. Some of the metabolic perturbations are extremely drastic to the fungal cells, which culminate in arresting nutrition, growth, proliferation, differentiation, adhesion, invasion and dissemination. In the present chapter, we will describe the beneficial effects of aspartic PIs against some human fungal pathogens, reporting in details their mechanisms capable in disturbing the fungal homeostasis.

Keywords Fungal infections • Alternative chemotherapy • Aspartic peptidases • Aspartic peptidase inhibitors • Development • Virulence

1 Fungal Infections

Fungi are heterotrophic and eukaryotic organisms that present two distinct cellular architectures: multicellular filamentous (e.g., hypha and pseudohypha) and single-celled cells (e.g., yeast and conidium). It is estimated that fungal kingdom contains about 1.5 million species, but less than 200 species are associated with human diseases [1]. In the past three decades, several studies have reported a significant increasing in the number of diseases caused by fungi (mycoses), which results in considerable morbidity and mortality that contribute to health-related costs [2, 3]. For example, the rate of sepsis due to fungi in the United States of America increased around 207 % during the period from 1979 through 2000 [4].

Infections caused by fungal pathogens lead to a wide range of diseases including allergies, superficial infections and invasive mycoses [2, 5]. Fungi are increasingly recognized as major pathogens in immunosuppressed patients, occurring in the setting of both self-generated and iatrogenic immunosuppression, human immunodeficiency virus (HIV) infection as well as individuals with other risk-factors (Table 1) [6–9]. In the absence of these factors, fungi cause mild, self-limited infections that typically involve mucocutaneous surfaces. *Candida* spp. and *Cryptococcus* spp. are the most frequently isolated yeasts in clinical practice. Unusual yeasts are also emerging in immunosuppressed patients as opportunistic human pathogens such as non-*albicans Candida* (e.g., *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida guilliermondii* and *Candida krusei*), *Trichosporon* spp., *Rhodotorula* and non-*neoformans* cryptococci species [10]. Other uncommon yeasts that may cause invasive disease in severely immunosuppressed patients include: *Geotrichum*, *Hansenula*, *Malassezia* and *Saccharomyces* [10, 11]. The most frequent filamentous

Table 1 Commonest risk factors that predispose individuals to fungal infections

Previous fungal colonization	Intravenous drug abuse
Disrupted anatomic barriers	Immobility
Patient age	Hospitalization or intensive care unit stay
Premature birth	Neutropenia
Low birth weight	Malnutrition
Hemodialysis	Total parenteral nutrition
Severe trauma	Mechanical ventilation
Use of corticosteroids	Recent abdominal surgery
Broad-spectrum antibiotic therapy	Fibrotic and cavitary lung disease
Radiation therapy	Autoimmune diseases
Cancer chemotherapy	Presence of graft-versus-host disease
Transplantation surgery	Central venous catheterization
Gastrointestinal tract perforation	Intravascular implants
Phagocyte dysfunction	Prosthetic device
Burns	Diabetes mellitus
Renal failure	HIV infection

fungi isolated are *Aspergillus* spp., but *Fusarium* spp., *Scedosporium*/*Pseudallescheria* spp., *Penicillium* spp., *Pneumocystis jiroveci* (formerly *Pneumocystis carinii*) and Zygomycetes have become increasingly in the clinic arena [7, 12].

Considering the significant morbidity and mortality associated with invasive fungal infections, it is particularly important to make best use of the available antifungal drugs for prophylaxis and treatment [13, 14]. Improved treatment options for invasive fungal infections have been developed during the last 20 years [8, 15, 16]. However, standard antifungal drugs are not predictably effective against emerging yeasts and filamentous fungi and may cause undesirable side effects [17]. These therapies can be limited also because of toxicity, long period of treatment, low efficacy rates and drug resistance [18–21]. The improvement of new antifungal compounds is very limited, since the fungal cells have several similarities to the mammalian cells, which have made the search for antifungal drug targets extremely difficult [22]. In addition, relatively little is known about the biochemical mechanisms of the pathogenicity of fungi as well as the virulence attributes directly implicated during the infectious processes [5, 23]. Recently, novel potential targets for antifungal drug development have been proposed [24–26], including proteolytic enzymes that play essential metabolic and regulatory functions in many central biological processes of fungal cells [27–31]. Consequently, fungal proteolytic enzymes, especially aspartic-type peptidases, play a variety of roles in establishing, maintaining and exacerbating an infectious process.

2 Proteolytic Enzymes

In the very early days, peptidases were considered principally as enzymes that cleave proteinaceous substrates [32]. Through many years, studies on peptidases were concentrated in the catabolic role of this class of enzymes. Nevertheless, this

view has changed drastically and peptidases are now gaining recognition as relevant signaling molecules participating in the control of multiple biological processes in all living organisms [33–41]. Through a high degree of specificity for substrates cleavage, peptidases are involved in the control of cell cycle progression, proliferation and death, tissue remodeling, hemostasis (coagulation) and in the immune response [42]. Consequently, the proteolytic activity regulation is strongly well-controlled (at transcriptional level by activation of zymogens, inhibitors or cofactors binding) to avoid inappropriate substrate cleavage.

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, the term ”peptidase” is recommended to be used as being synonymous of ”peptide hydrolase” for any enzyme that hydrolyses peptide bonds. Therefore, peptidases (EC 3.4) are classified in subclass 4 of class 3 (hydrolases). Also called proteases or proteolytic enzymes, peptidases were primarily classified into endopeptidases, which cleave internal peptide bonds, and exopeptidases, which the action is directed from the amino (NH_2) or carboxy (COOH) termini of the corresponding proteinaceous substrate [43–48]. Based on their site of action, the exopeptidases that act only near the free amino terminus and liberate a single amino acid residue, a dipeptide or a tripeptide are classified as aminopeptidases, dipeptidyl peptidases or tripeptidyl peptidases, respectively. The exopeptidases acting at a free carboxy terminus liberate a single amino acid (carboxypeptidases) or a dipeptide (peptidyl dipeptidases). Additionally, peptidases can be divided according to the essential catalytic amino acid residues at their active sites—serine, glutamic, threonine, cysteine and aspartic peptidases—or even metal, metallopeptidases (Fig. 1a). However, there are some peptidases that do not fit into the proposed classification [49–55].

All peptidases bind their substrates in a groove or cleft, where peptide bond hydrolysis occurs. Schechter and Berger [56] defined that the active site residues in the peptidase are composed of contiguous pockets termed subsites. So, amino acid side chains of substrates occupy the enzyme subsites in the groove, labeled as …-S4-S3-S2-S1-S1′-S2′-S3′-S4′-… that bind to corresponding substrate residues …-P4-P3-P2-P1-P1′-P2′-P3’-P4′-… with respect to the cleavable of peptide bond (Fig. 1b).

Rendering to MEROPS database (http://www.merops.sanger.ac.uk), the subsubclasses of peptidases are grouped in families according to a significant similarity in amino acid sequence. For classification, each family is identified by a letter representing the catalytic type of the peptidase, followed by a unique number. Families that have arisen from a single evolutionary origin of peptidases and are homologous are grouped in clans [57]. Each clan is symbolized by two letters, in which the first one is representing the catalytic type of the family. Thus, a clan represents one or more families that show evidence of their evolutionary relationship by their similar tertiary structures, by the order of catalytic site residues in the polypeptide chain and by common sequence motifs around the catalytic residues [55].

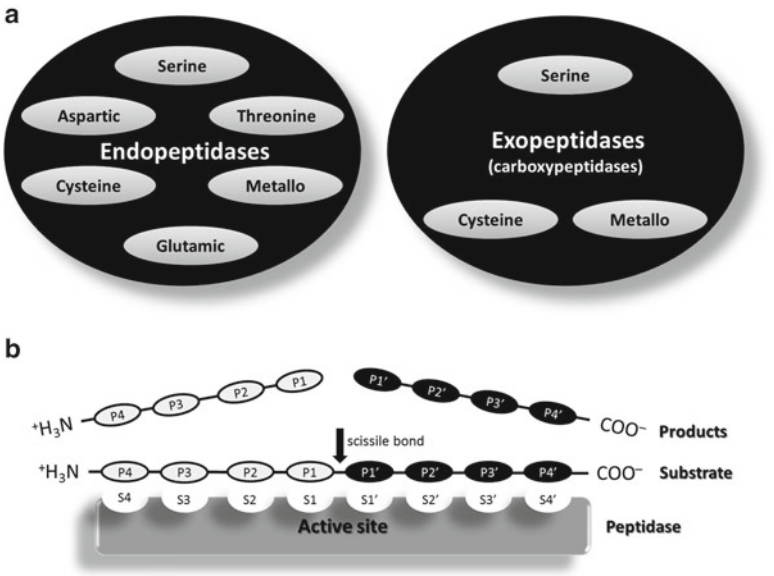

Fig. 1 (**a**) General classification of both endopeptidases and exopeptidases, specifically carboxypeptidases, according to the chemical group present in their catalytic sites. (**b**) Schematic representation of binding region and catalytic site of a peptidase. This hypothetical peptidase possesses eight subsites (S1–S4 and S1′–S4′) in its catalytic site and, consequently, is able to recognize and bind to a sequence of eight amino acids (P1–P4 and P1′–P4′) in the substrate. After proteolysis, at least two smaller peptides are generated as the reaction products

3 Aspartic Peptidases

The aspartic peptidases are exclusively endopeptidases, with low optimal pH values for best activity of hydrolysis. They are classified into five clans (AA, AC, AD, AE and AF), according to the MEROPS database [57], which comprise 14 families and 16 subfamilies distributed only in clans AA and AD (Table 2). However, there are two families with unassigned clans (Table 2). Each clan represents one or more families that show evidence of their evolutionary relationship, similar tertiary structures, order of catalytic site residues in the polypeptide chain and their common sequence motifs around the catalytic site [57].

In general, aspartic peptidases are characterized by two aspartic carboxyl groups as key catalytic groups at their active site (Asp32 and Asp215 in the porcine pepsin sequence numbering) [58–60] and a scission preference between large and hydrophobic amino acids [61–63]. Despite the fact that most aspartic peptidases fit in these characteristics, substantial variances exist according to catalytic properties, cellular localization, biological functions and inhibition by pepstatin A (isovaleryl-Val-Val-Sta-Ala-Sta-OH) (Fig. 2), a classical aspartic proteolytic inhibitor, which is

Table 2 MEROPS classification of aspartic-type peptidases

Clan	Family	Subfamily
AA	A1	A1A, A1B
	A2	A2A, A2B, A2C, A2D, A2E, A2G
	A3	A3A, A3B
	A9	–
	A11	A11A, A11B
	A28	–
	A32	–
	A33	–
AC	A8	–
AD	A22	–
	A24	A24A, A24B
AE	A25	–
	A31	–
AF	A26	–
Unassigned	A5	–
Unassigned	A36	–

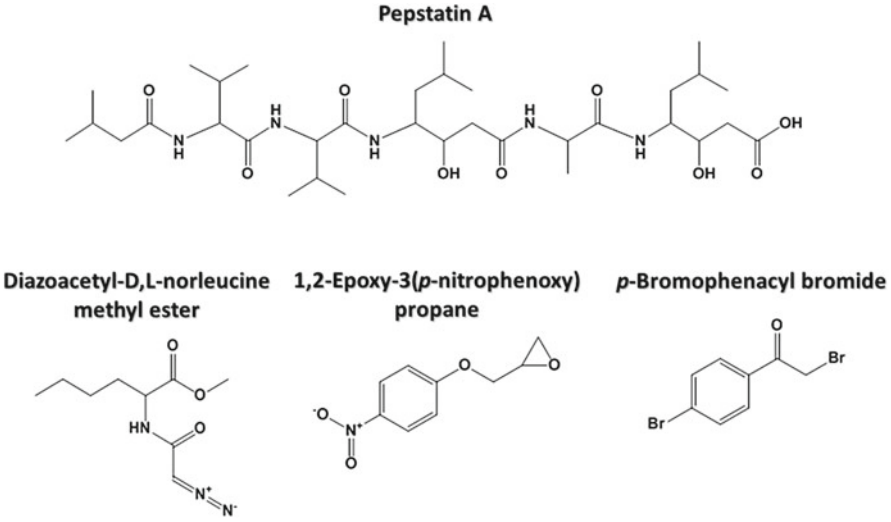

Fig. 2 Classical aspartic peptidase inhibitors

a naturally occurring microbial hexapeptide containing the unusual amino acid statine (Sta, (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid) [64]. Also, diazoacetyl-D,L-norleucine methyl ester, *p*-bromophenacyl bromide and 1,2-epoxy-3-(*p*-nitrophenoxy) propane have been used as diagnostic reagents for aspartic peptidases (Fig. 2).

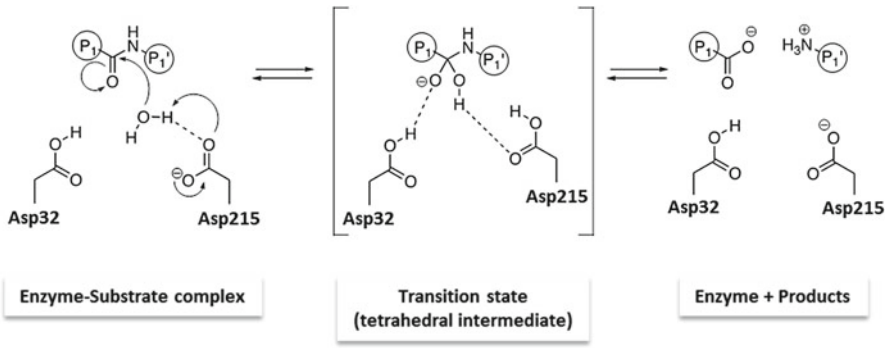

Fig. 3 Catalytic mechanism of aspartic peptidases. A water molecule tightly bound to the aspartates in the native enzyme is proposed to nucleophilically attack the scissile bond carbonyl. The resulting tetrahedral intermediate is stabilized by hydrogen bonds to the negatively charged carboxyl of Asp32. Fission of the scissile C–N bond is accompanied by transfer of a proton to the leaving amino group either from Asp215. *Dashed lines* indicate hydrogen bonds

Aspartic peptidases are synthesized as zymogens, which are converted to the active form by acid-triggered, autocatalytic proteolysis and removal of lengths of polypeptides that are *N*-terminal extensions [65, 66]. Most eukaryotic aspartic peptidases are monomeric and consist of a single polypeptide chain that forms two similar domains with the active site cleft located between them; each domain provides an aspartic carboxyl group as key catalytic group to form the active site [51, 61, 67–70]. In the active enzyme, the two aspartic residues are geometric closer and one aspartate is ionized, whereas the second one is unionized at the optimal pH [62, 71]. The most widely accepted mechanism of action of the aspartic peptidases is a general acid-base catalysis involving coordination of a water molecule between the two highly conserved aspartate residues (Fig. 3). One aspartate activates the water by abstracting a proton, enabling the water to attack the carbonyl carbon of the substrate scissile bond, generating a tetrahedral oxyanion intermediate. Rearrangement of this intermediate leads to protonation of the scissile amide. Restabilizing from the transition state, the amino moiety from the substrate becomes a better leaving group, and the substrate is cleaved [72–77] (Fig. 3).

4 Fungal Aspartic Peptidases as Promising Chemotherapeutic Targets: An Overview

The pathogenesis of fungal infections is facilitated by the expression of a number of virulence factors, including hydrolytic enzymes. Aspartic peptidases produced by human fungal pathogens (Table 3) play fundamental roles in distinct processes, both at the physiological and infective levels, such as nutrition, growth, proliferation, cell

Table 3 Fungal aspartic peptidases, deposited in the MEROPS database, belong to the clan AA, family A1 and subfamily A

	MEROPS classification	
Fungal species	Peptidase name	Peptidase identifier
Aspergillus fumigatus	Peptidase F	A01.026
Aspergillus niger	CtsD peptidase	A01.077
	PepAa peptidase	A01.079
	PepAb peptidase	A01.080
	PepAc peptidase	A01.081
Aspergillus oryzae	Oryzepsin	A01.072
Aspergillus saitoi	Aspergillopepsin I	A01.016
Candida albicans	Candidapepsin Sap1	A01.014
	Candidapepsin Sap2	A01.060
	Candidapepsin Sap3	A01.061
	Candidapepsin Sap4	A01.062
	Candidapepsin Sap5	A01.063
	Candidapepsin Sap6	A01.064
	Candidapepsin Sap7	A01.065
	Candidapepsin Sap8	A01.066
	Candidapepsin Sap9	A01.067
	Candidapepsin Sap10	A01.085
Candida parapsilosis	Candiparapsin (Sapp1)	A01.038
	Sapp2p peptidase	A01.076
Candida tropicalis	Canditropsin (Sapt)	A01.037
Cryphonectria parasitica	Endothiapepsin	A01.017
Cryptococcus neoformans	CnAP1 peptidase	A01.078
Penicillium janthinellum	Penicillopepsin	A01.011
Podospora anserina	Podosporapepsin	A01.044
Rhizopus microspores	Rhizopuspepsin	A01.012
Rhizomucor miehei	Mucorpepsin	A01.013
Saccharomyces cerevisiae	Barrierpepsin	A01.015
	Saccharopepsin	A01.018
	Yapsin-1	A01.030
	Yapsin-2	A01.031
	Yapsin-3	A01.035
	GPI-anchored aspartic peptidase	A01.A61
	Yapsin-6	A01.A62
Schizosaccharomyces pombe	Yps1 protein	A01.056
	Sxa1 peptidase	A01.A91
Syncephalastrum racemosum	Syncephapepsin	A01.042
Trichoderma reesei	Trichodermapepsin	A01.027
	SA76 peptidase	A01.082
Yarrowia lipolytica	Axp peptidase	A01.036

cycle progression, signaling, death pathways, cellular differentiation, adhesion to both abiotic and biotic substrates, host cell invasion, catabolism of host proteins and both stimulation and evasion of host immune responses [28–31, 68, 78–87] (Fig. 4).

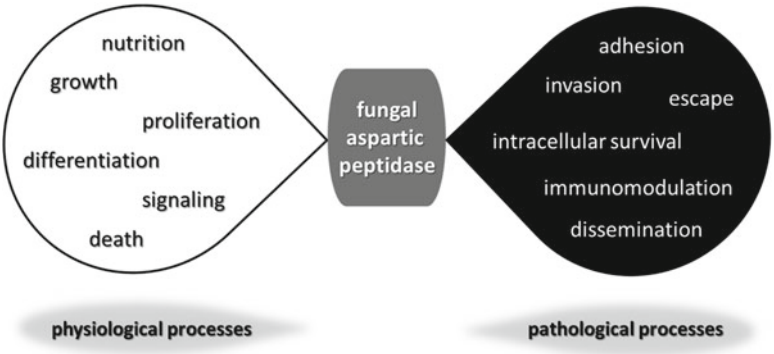

Fig. 4 Possible roles played by fungal aspartic peptidases in both physiological and pathological processes. Aspartic proteolytic inhibitors are able to block one or several of these fundamental events, reducing the ability of the fungus in causing infections

A number of fungi (Table 3) are able to produce both surface and/or secreted aspartic peptidases with multifunctional properties, which are expressed during different phases of their life cycles. Interestingly, but quite alarming, some fungal pathogens, in particular those belonging to the genus *Candida*, are able to overexpress aspartic peptidases when exposed to subinhibitory concentrations of classical antifungals belonging to the azole, echinocandin and pyrimidine analogue classes [88–93]. As consequence, fungal aspartic-type peptidases are prospective targets for the development of potent and effective antifungal drugs. Corroborating these findings, several scientists around the world have devoted efforts to elucidate the three dimensional structures of fungal aspartic peptidases in order to find and/or synthesize specific proteolytic inhibitors. In this sense, peptidase-inhibitor crystal structures are available on the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) or PubMed (http://www.pubmed.com) databases for some fungal aspartic peptidases, including aspergillopepsin from *Aspergillus phoenicis*, *Aspergillus niger* and *Aspergillus oryzae*; endothiapepsin from *Cryphonectria parasitica*; mucorpepsin from *Rhizomucor miehei*; penicillopepsin from *Penicillium janthinellum*; pepsin from *Rhizopus pusillus*; rhizopuspepsin from *Rhizopus microsporus* var. *chinensis*; saccharopepsin from *Saccharomyces cerevisiae*; secreted aspartic peptidase 1 (Sap1), Sap2, Sap3 and Sap5 from *C. albicans*; Sapp1 from *C. parapsilosis*; Sapt from *C. tropicalis* and trichodermapepsin from *Trichoderma reesei*.

5 Aspartic Peptidase Inhibitors: Antifungal Properties

Peptidase inhibitors (PIs) block the proteolytic activity by preventing the substrate access to the active site. In competitive inhibition, the inhibitor binds directly to the active site, thus preventing enzyme-substrate interaction. In non-competitive

Table 4 Current licensed HIV PIs

Generic name	Trade name	Abbreviation	Manufacturer	FDA approval
Amprenavir	Agenerase	APV	GlaxoSmithKline	1999 (April)
Atazanavir	Reyataz	ATV	Bristol-Meyers Squibb Company	2003 (June)
Darunavir	Prezista	DRV	Tibotec Pharmaceuticals	2006 (June)
Fosamprenavir	Lexiva	F-APV	GlaxoSmithKline	2003 (October)
Indinavir	Crixivan	IDV	Merck	1996 (March)
Lopinavir	Kaletra	LPV	Abbott Laboratories	2000 (September)
Nelfinavir	Viracept	NFV	Pfizer	1997 (March)
Ritonavir	Norvir	RTV	Abbott Laboratories	1996 (March)
Saquinavir	Invirase[a] Fortovase[b]	INV	Roche	1995 (December)
Tipranavir	Aptivus	TPV	Boehringer Ingelheim Pharmaceuticals	2005 (June)

[a] Hard
[b] Soft gelatin capsules

inhibition, the inhibitor binds to an allosteric site, which alters the active site and makes it inaccessible to the substrate [54, 94]. Efforts to achieve pharmacological inhibition of fungal aspartic peptidases were fruitfully paralleled by the development of active compounds capable in inhibiting the HIV-1 aspartic peptidase (amprenavir, atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir and tipranavir) (Table 4 and Fig. 5), since these HIV PIs presented both *in vitro* and *in vivo* antifungal properties [28–31, 68, 78–87].

5.1 Clinical Evidences

Findings from clinical trials reported that the introduction of HIV PIs in the treatment of HIV-infected individuals resulted in a noticeable decrease on the opportunistic infections caused by fungi, mainly by species belonging to the following genera: *Candida*, *Cryptococcus* and *Pneumocystis*. For instance, a multinational cohort study with 6,941 HIV-positive individuals from Australia and 10 European countries, comparing the periods 1997–2001 and 1994–1996, revealed a significant HIV PI-induced decrease in the progression of candidiasis from 17.0 % to 5.7 % [95]. In accordance, Hoegl et al. [96] published a study over a period of 2 years that revealed a drastic reduction on the frequency and the severity of mucosal candidiasis in HIV-infected patients [96]. The usage of HIV PIs on clinical practices also promoted a significant changing on the epidemiology of cryptococcosis among individuals with HIV infection, as reported in two distinct hospitals from metropolitan areas of United States of America: Atlanta area ranging from 66 cases per 1,000 persons in 1992 to 7 cases per 1,000 in 2000 and Houston area ranging from 23.6 cases per 1,000 persons in 1993 to 1.6 cases per 1,000 persons in 2000 [97]. Similarly, the incidence of *Pneumocystis* pneumonia declined from a high of approximately 90 infections per 1,000 person-years in 1995, prior to the introduction of PI in the cocktail offered to HIV-positive individuals, to approximately 30 per 1,000 person-years in 2001 [98, 99].

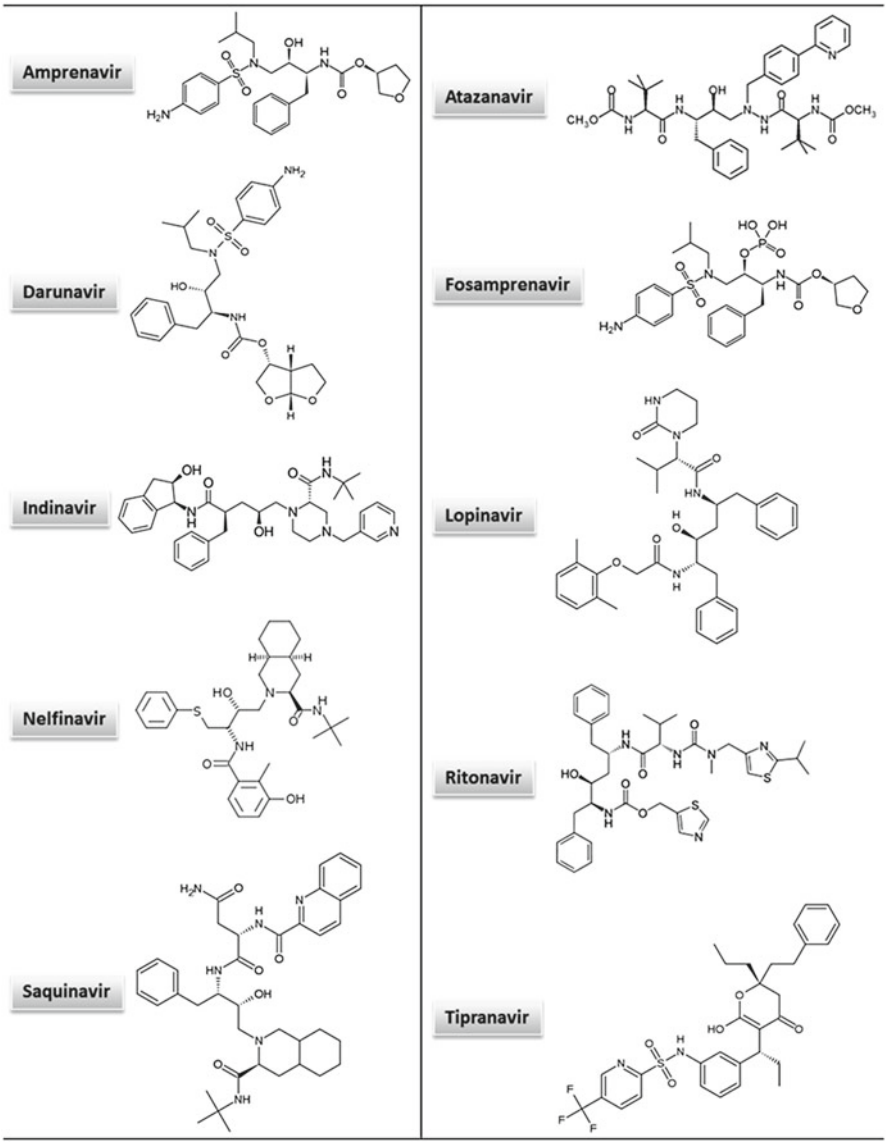

Fig. 5 Chemical structures of the current licensed HIV PIs

5.2 Proteolytic Activity Inhibition

Experimental studies suggested that elevated aspartic peptidase production is an important aspect of fungi interaction with host tissue as well as strain virulence [100–107]. In theory, the inhibition of aspartic peptidase activities can interfere with the biological events regulated by these multifunctional enzymes (Fig. 4).

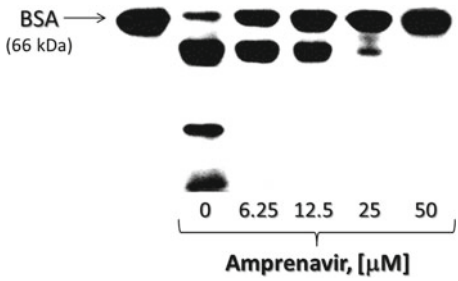

Fig. 6 Effects of amprenavir on the Sap2 activity produced by *C. albicans*. Sap2 was incubated for 1 h at 37 °C in 20 mM sodium citrate, pH 4.0, containing 0.1% bovine serum albumin (BSA) in the absence or in the presence of different concentrations of amprenavir. After that, the BSA cleavage by Sap2 as well as the inhibition profile was shown through sodium dodecyl sulfate polyacrylamide gel electrophoresis. The migration profile of BSA diluted in sodium citrate buffer is shown in the first slot

Borg von Zepelin et al. [108] demonstrated that purified Sap1, Sap2 and Sap3 of *C. albicans* were inhibited by the first generation of HIV PIs, ritonavir, saquinavir, indinavir and nelfinavir, in a concentration-dependent manner, in which ritonavir was the most effective inhibitor of these Sap isoenzymes. In contrast, only a slight inhibition of proteolytic activity was detected in purified Sap4, Sap5 and Sap6. Similar results were published in parallel by other distinct research groups [109, 110]. Recently, Braga-Silva et al. [111] compared the effect of the first and second generations of HIV PIs on Sap2, which is the main pronounced virulence factor expressed during the infection processes by *C. albicans*. The results showed that amprenavir was found to be the most potent inhibitor of Sap2, reducing the activity by approximately 92 % at 100 μM, while the other HIV PIs tested (ritonavir, saquinavir, indinavir, nelfinavir and lopinavir) inhibited around 45 %–65 % of the proteolytic activity [111]. As illustrated in the Fig. 6, amprenavir inhibited the aspartic peptidase activity secreted by *C. albicans* in a characteristic dose-dependent fashion.

The search for new, more potent and more selective PIs able to block fungal aspartic peptidases have led several groups to synthesize novel compounds, especially belonging to the peptidomimetic class. For instance, Skrbec and Romeo [112] tested the effects of nineteen novel peptidomimetic compounds, which were synthesized based on the transition-state of the aspartic peptidase of HIV-1, on Sap2. Ten of those compounds exhibited IC_{50} values against Sap2 lower than 15 μM, in which the best inhibitor, Kyn-Val-Phe-Psi[OH-OH]-Phe-Val-Kyn, blocked the hydrolysis of albumin contained in the culture medium, arresting the *C. albicans* development.

Pichová et al. [113] compared the inhibition profile of purified Saps from *C. albicans* (Sap2), *C. tropicalis* (Sapt1), *C. parapsilosis* (Sapp1) and *C. lusitaniae* (Saplu) with ritonavir, saquinavir, nelfinavir and indinavir. The results demonstrated that the best HIV PI from this set was ritonavir that inhibited all four peptidases in micromolar range. The K_i values of saquinavir for Sap2, Sapp1 and Saplu were one order of magnitude higher. Sapt1 was inhibited with similar potency by both saquinavir and ritonavir. Indinavir inhibited only the Sapt1 and nelfinavir did not inhibit

Table 5 Differential responsiveness of aspartic peptidases secreted by conidial and mycelial forms of *F. pedrosoi* to different HIV PIs

Fungal form	Optimum pH	Total enzymatic activity (arbitrary units)	% Proteolytic activity			
			Indinavir	Saquinavir	Ritonavir	Nelfinavir
Conidia	4.0	42.7 ± 6.5	28.9 ± 8.0	29.9 ± 4.8	41.9 ± 6.7	7.3 ± 2.3
Mycelia	2.0	113.5 ± 8.1	2.6 ± 1.2	71.6 ± 3.8	3.3 ± 2.1	10.3 ± 5.2

any of these peptidases [113]. In other study, Hrusková-Heidingsfeldová et al. [114] compared the inhibition of purified Sapp1 and Sapp2 by the HIV PIs and ritonavir was the best compound, inhibiting more efficiently Sapp2 in submicromolar concentration ($K_i = 0.3$ μM) than Sapp1 ($K_i = 1.9$ μM).

The cultivation of *C. neoformans* cells for 48–72 h in the presence of 10 μM indinavir or 0.1 μg/mL pepstatin A considerably diminished the extracellular peptidase activity measured in the cell-free culture supernatant in comparison with yeast grown in the absence of these PIs [115]. Conversely, the recombinant aspartic peptidase (CnAP1) from *C. neoformans* did not alter its hydrolytic activity behavior in the presence of three different HIV PIs (indinavir, lopinavir and ritonavir) up to 25 μM, whereas the classical aspartic PI pepstatin A reduced its enzymatic activity of approximately 87 % in respect to the control [116]. The simplest explanation for this discrepancy is that CnAP1 is not the direct target of these anti-HIV drugs, and that other aspartic peptidases existing in *C. neoformans* represent the real target. Scanning the *C. neoformans* cDNA database, Pinti et al. [116] found other gene encoding a putative aspartic peptidase (*cnap2*).

Conidial and mycelial cells of *Fonsecaea pedrosoi*, the major etiologic agent of chromoblastomycosis, were able to produce extracellular aspartic peptidases when grown under chemically defined conditions. Both aspartic peptidases were active at acidic pH, capable in cleaving several human proteinaceous substrates and fully sensitive to pepstatin A at 10 μM [105–107]. The effects of saquinavir, ritonavir, indinavir and nelfinavir on secreted aspartic peptidases of *F. pedrosoi* cells were also evaluated. As results of those experiments, a typical dose-dependent inhibition was observed for all HIV PIs [105, 107]. However, the aspartic peptidases released by both *F. pedrosoi* morphotypes had distinct biochemical properties including optimum pH for reaching their maximal activities as well as sensibility to HIV PIs [105–107] (Table 5).

5.3 Molecular Modeling Studies

The established ability of pepstatin A, HIV PIs and peptidomimetic compounds to inhibit fungal aspartic peptidases and the availability of crystallographic three-dimensional structures for a number of these proteins have triggered a few molecular modeling studies. These efforts have been mostly restricted to molecular docking calculations aimed to unveil how these molecules bind to a given aspartic peptidase

comparatively to HIV-1 peptidase [117–119]. Molecular docking methods provide an approach to the ranking of potential ligands with respect to their ability to interact with a given target [120–124]. It involves efficient sampling of possible poses of the small molecules in the specified binding pocket of a protein in order to identify the optimal binding geometry, as measured by a score function [125–128].

PIs and HIV-1 peptidase exhibit a pattern where a centrally located hydroxyl group in the PIs chain forms a hydrogen bond with the catalytic residues Asp25/Asp25′, as illustrated by the ritonavir-HIV peptidase complex in Fig. 7a. The complex is further stabilized by non-specific electrostatic interactions with the protein backbone and hydrophobic contacts with protein side chains surrounding the ligand in the active site. While this pattern seems to be common in Saps, differences in their active site render quite different inhibitory activities for the same compounds. As an example, ritonavir and saquinavir have similar K_i against HIV-1 peptidase (nM range) [129, 130]; however, ritonavir has one order of magnitude higher affinity for Sap2 of *C. albicans* than saquinavir (μM range) [113]. To address the rationale behind these distinct inhibitory potential, docking calculations have been carried out to identify the differences in binding mode of the ligands to Sap2 of *C. albicans* [117]. A main difference observed in the binding mode of these two ligands is that only ritonavir occupies the protein subsite S3. Based on structure of Sap2 of *C. albicans* complexed with inhibitor A70450 (a synthetic hexapeptide analogue to pepstatin A), it has been proposed that binding to S3 region is critical for the efficient inhibition of this protein [119]. Therefore, the results from the molecular docking offer a potential explanation for the different inhibitory potential, corroborating this hypothesis. On the other hand, theoretical studies have indicated that ritonavir does not interact with the catalytic residues Asp32/Asp218 of Sap2 (as Asp25/Asp25′ in HIV-1 peptidase). However, a recent (and to date unpublished) structure determined by X-ray crystallography of ritonavir bound to Sap2 reveals that this interaction does occur along with other similar interactions between ritonavir and HIV-1 peptidase.

Despite of preserved interactions upon binding of the same ligand to different aspartic peptidases, even subtle structural differences in the active site can affect the ligand binding mode, and hence its inhibitory efficiency. A comparison of the binding of ritonavir in the active sites of Sap2 of *C. albicans*, Sapp1 of *C. parapsilosis* and the HIV-1 peptidase is presented here as an example (Fig. 7a, b). As for Sapp1, ritonavir interacts with the catalytic dyad Asp32/Asp218 in Sap2, (Asp32/Asp220 in Sapp1), binds its isopropylthiazol group to S3, the thiazol group to S1′, the two benzyl groups to S1 and S2′ and its isopropyl group to S2 (Fig. 7c). While many interactions are common to all three proteins, the different electrostatic and hydrophobic contributions in each active site will lead to significantly different inhibitory potential.

Although previous molecular docking studies have highlighted key structural information that can be potentially useful for the design of selective Sap inhibitors, knowledge in this area remains scarce. The use of additional molecular modeling techniques, such as sampling-based methods, can offer quantitative insights into the binding energy contributions underlying the differential inhibitory activities of Sap inhibitors. The integration between a variety of computational approaches and

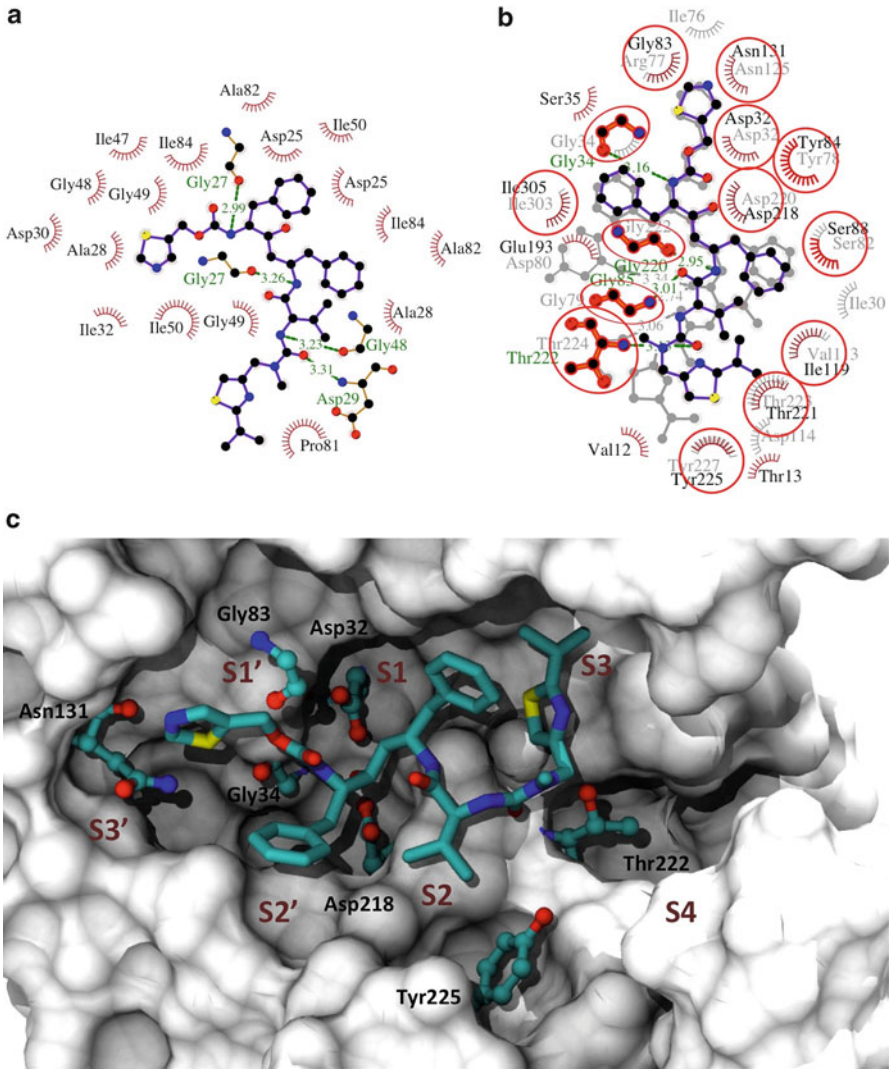

Fig. 7 Comparison of the binding mode of ritonavir to active sites of (**a**) HIV-1 peptidase and (**b**) Sap2 of *C. albicans* (*color*) and Sapp1 of *C. parapsilosis* (*grey*). Electrostatic interactions between ligand and protein are shown as *dotted lines*. Hydrophobic contacts are represented by *spiked semi-circles*. The residues involved in each interaction are marked by their three-letter code and number in each protein. Atoms are color coded as follows: carbon, *black*; oxygen, *red*; nitrogen, *blue*; sulfur, *yellow*. (**c**) Three-dimensional representation of ritonavir bound to Sap2 of *C. albicans*. Sap2 is represented by its molecular surface; surface area corresponding to flap region (residues 71 to 89) and displayed interacting residues is omitted for clarity. Ritonavir is shown as stick model and interacting residues in CPK model. (Atoms are color coded as follows: carbon, *cyan*; oxygen, *red*; nitrogen, *blue*; sulfur, *yellow*). Subsite binding regions are labeled in *dark red*; displayed residues are identified by their three-letter code and corresponding sequence number. Rendering was produced using the VMD program [131] starting from coordinates of PDB ID 3Q70 [Koester, Heine and Klebe, unpublished work]

experimental measurements is expected to bring forward a deeper understanding of the factors modulating the interaction of aspartic peptidases and potential inhibitors, and lead to novel compounds with improved inhibitory efficiency.

5.4 Anti-Proliferative Action

The blockage of secreted and/or surface aspartic peptidases can result in an incapability of the fungal cells in obtaining peptides and amino acids to its suitable nutrition, leading to a reduction or even a complete interruption in the cellular development. Several studies in the literature have reported the potential role of classic inhibitors of aspartic peptidases (Fig. 2) as well as HIV PIs (Fig. 5) on the growth and/or proliferation of different human pathogenic fungi [28–31, 68, 78–87].

In *C. albicans*, numerous viability methods such as colony formation, mitochondrial activity (e.g., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay) and membrane permeability (e.g., propidium iodide incorporation) assays were applied in order to show that pepstatin A, indinavir, saquinavir, ritonavir and amprenavir (Fig. 8), in different extensions and ways, were able to significantly reduce the *in vitro* growth of *C. albicans* yeast cells [108–111, 132–134]. As common findings, the aspartic PIs tested noticeably blocked the development of *C. albicans* in a typical cell- and drug-concentration inhibition mechanism when the yeasts were cultivated in a medium containing albumin as the sole nitrogen source and at acidic pH condition. This inhibition phenomenon probably is due to the interference of the PIs with Saps, preventing the assisted nitrogen assimilation. On the contrarily, cultivation of yeasts in nitrogen-unlimited complex media (e.g., brain heart infusion and Sabouraud), where Sap expression is not required for growth, the PIs did not arrest the *C. albicans* proliferation [87, 135, 136].

Nanomolar and subnanomolar K_i values of pepstatin A and six pepstatin analogs efficiently inhibited the proteolysis and growth of different clinical isolates of *C. albicans*, *C. tropicalis* and *C. parapsilosis* when tested on hemoglobin-supplemented medium [137]. In an aspartic peptidase-inducing medium, Sapp activity in 20 clinical isolates of *C. parapsilosis* was observed at 37 °C but not at 22 °C, and the presence of ritonavir (at 8 μg/mL) promoted an inhibition of approximately 50 % in the albumin consumption and also delayed yeast growth; however, saquinavir did not have any effect either on growth or on Sap activity [138].

Pepstatin A and indinavir reduced the growth of ATCC and clinical isolates of *C. neoformans* in both dose- and time-dependent manner. For instance, these two aspartic PIs did not impair *C. neoformans* development before 48 h of incubation, but a significant decrease was observed after this time [115, 139].

The aspartic PIs were also tested on the ability to interfere in the growth behavior of filamentous fungi including *F. pedrosoi* and *P. jiroveci*. In the former, conidial cells were incubated for 1 h with pepstatin A, nelfinavir, saquinavir, indinavir and ritonavir and the results revealed a fungicidal action directly dependent of both cell number and inhibitor concentration, being pepstatin A and saquinavir the best

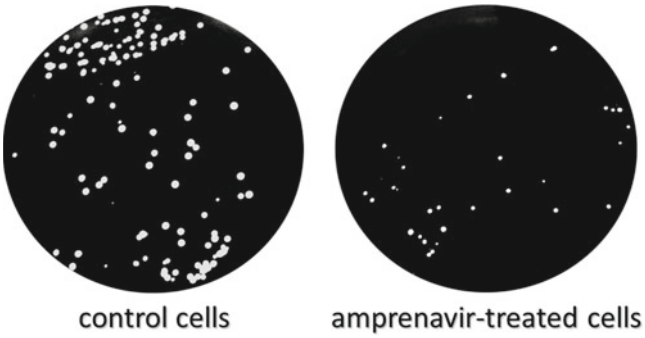

Fig. 8 Development of *C. albicans* on plates containing yeast carbon base medium supplemented with 0.1% bovine serum albumin (YCB-BSA). The yeast cells were pre-treated without (control cells) or with 100 **μ**M amprenavir for 20 h and then plated on YCB-BSA. Amprenavir induced a drastic reduction on the numbers of colony-forming units. Note that the yeast cells pre-incubated with amprenavir have generated much smaller colonies on agar when compared to untreated cells

compounds able to significantly inhibit the viability of conidia (10^3 cells) at the concentration of 0.1 μM and 50 μM, respectively [105, 107]. Because of the lack of a continuous *in vitro* culture system for *P. jiroveci*, the effects of pepstatin A, ritonavir, saquinavir, indinavir, nelfinavir, amprenavir, tipranavir and lopinavir on this fungal viability were assayed using monoxenic culture of human embryonic lung cells infected by *P. jiroveci* that was previously isolated from infected rat lungs. All the tested PIs were able to block the development of *P. jiroveci* at concentrations clinically achievable *in vivo* [140–145].

5.5 Morphological Alterations

Few studies have reported the effect of aspartic PIs on the morphology and/or ultrastructure of fungal cell. For instance, the colonies of *C. albicans*, *C. tropicalis* and *C. parapsilosis* affected by the presence of pepstatin A were much smaller and less frequent than those growing on a control plate without inhibitor [137]. Similar results were reported by Braga-Silva and Santos [87] after the treatment of *C. albicans* cells with ritonavir or amprenavir (Fig. 8). Validating these discoveries, amprenavir promoted a significant reduction (around 30 %) on the yeast cell size when compared with amprenavir-untreated cells as revealed by flow cytometry measurements. In this same line of thinking, a simple inspection by means of optical microscopy observations of *C. albicans* cells treated with amprenavir have revealed some clear morphological alterations in comparison to the typical appearance of yeasts, including non-ovoid, petite and lysed cells (Fig. 9). Braga-Silva et al. [111] also reported alterations at the ultrastructural levels after treatment of *C. albicans* yeast cells with different concentrations of amprenavir. The ultrastructure of amprenavir-treated cells showed alterations in the shape and cell surface sculpturing, such as invaginations at

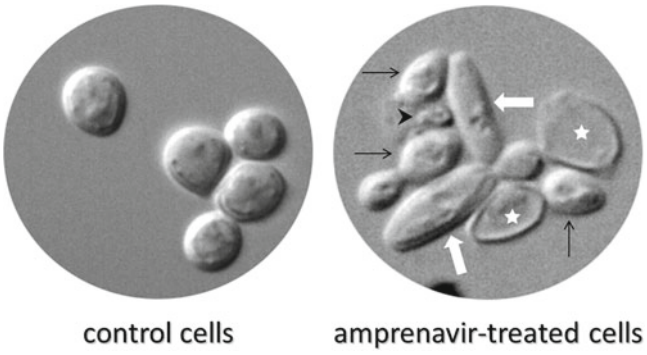

Fig. 9 Optical microscopy exemplifying the morphological differences observed in *C. albicans* yeast cells grown in yeast carbon base liquid medium containing 0.1% bovine serum albumin in the absence (control cells) or in the presence of 100 **µ**M amprenavir for 48 h. The control cell population is homogeneous and consists of rounded to oval yeast cells. Contrarily, amprenavir induces some clear morphological alterations such as petite cells (*arrowhead*), smaller cells (*black arrows*), large non-ovoid cells (*white arrows*) and ghost or lysed cells (*asterisks*)

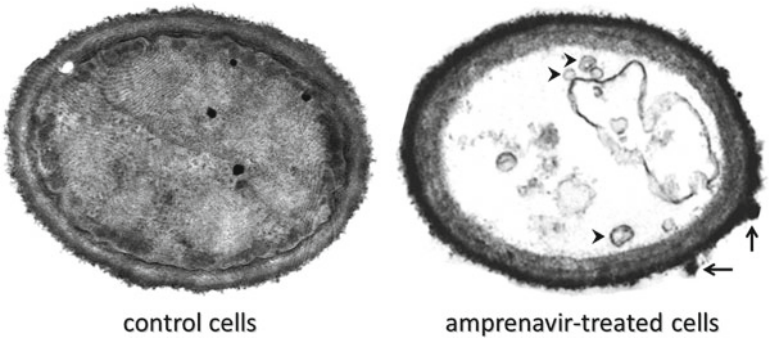

Fig. 10 Transmission electron microscopy showing some ultrastructural alterations observed in *F. pedrosoi* conidial cells after treatment with 100 **µ**M saquinavir for 1 h. Control cells present a characteristic dense cytoplasm with a distinct and compact cell wall. In contrast, saquinavir-treated cells present disorder and detachment of cell wall (*arrows*), vesicles in cytoplasm (*arrowheads*) and rarefaction of cytoplasm, exemplifying a typical necrotic cell

the surface, as observed by means of scanning electron microscopy. The surface of control yeasts had numerous protrusions, while cells treated with amprenavir demonstrated a smooth surface due to the loss of the irregular surface layer.

Transmission electron microscopy investigations demonstrated that both nelfinavir and saquinavir at 100 µM induced several ultrastructural alterations in *F. pedrosoi* conidial cells (Fig. 10), including: invaginations in the cytoplasmic membrane and withdrawal of the cytoplasmic membrane from within the cell wall, disorder and detachment of the cell wall, rupture of internal organelles, and the presence of

large and irregular cytoplasmic vacuoles. Furthermore, abnormal division disfiguring regular conidia morphology could be observed in cells treated with nelfinavir, while the saquinavir treatment seams to induce breakage of cell wall starting from extracellular environmental. Ritonavir and indinavir also promoted some of these aberrant alterations; however, in a lower proportion when compared to nelfinavir and saquinavir treatment [107]. All the irreversible effects observed on the morphological architecture powerfully corroborate the anti-proliferative properties of the aspartic PIs on fungal growth and development.

5.6 Modulation of Virulence Attributes

The ability of fungal cell to modulate its metabolism as well as to produce virulence factors robustly contribute to the initiation, development and consolidation of a successfully infectious process. Fungal virulence factors can be divided into two major categories: molecules and/or structures that promote fungal colonization (e.g., adaptation to different environmental conditions such as pH, temperature, oxygen concentration, osmolarity and nutrient availability; contacting, adherence and invasion of host cells and structures; resistance to physical removal; competition for nutrients; resistance to the innate immune defenses such as phagocytosis, action of complement system and antimicrobial peptides; and evasion of adaptive immune defenses such as antibody) and damage to the host (e.g., toxins and hydrolytic enzymes like peptidases, lipases and hemolysins) [146, 147]. As an expected consequence, the blockage of either expression or production of fungal virulence attributes will help to minimize the damages/injuries in host organism, contributing to the containment of the pathogen. So, a drug able to inhibit these virulence attributes can be considered as a promising and potent pharmacological compound.

5.6.1 Morphogenesis

Changing cell size and shape are strategies employed by many fungi to survive in the environment and also within the host [148, 149]. More than this, cell differentiation is an essential step to the establishment of a successful infection by several human fungal pathogens. The understanding of this phenomenon and, consequently, how this morphological transition can be inhibited may allow the design of alternative and efficient therapies against fungal infections [148, 150]. Moreover, some works have reported the differential expression of aspartic peptidases during the fungal morphogenesis phenomenon, mainly in *C. albicans* [104, 151] (Fig. 11), and the usage of aspartic PIs can control this event. In this context, Gruber et al. [132] described that the ability to form hyphae was only delayed in indinavir-treated *C. albicans* yeast cells. Contrarily, amprenavir was able to arrest the yeast into germ tube differentiation in *C. albicans* (Fig. 11), showing a typical dependent drug dose inhibition [87, 111].

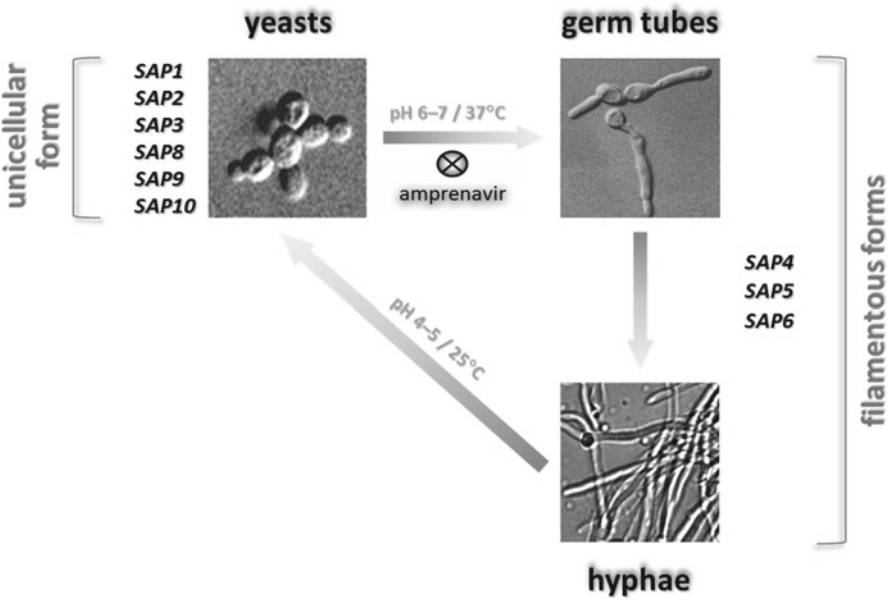

Fig. 11 Morphological stages of *C. albicans* and differential expression of *SAP* genes. Growth conditions such as temperature and pH directly influence the transition of yeasts into hyphae as well as the expression of *SAP* genes. In this context, the expression of *SAP1* to *SAP3* and *SAP8* to *SAP10* has been detected in yeast cells, while *SAP4* to *SAP6* has been associated with the filamentous forms. Amprenavir is able to block (⊗) the morphological transition in *C. albicans*

5.6.2 Secreted Molecules

Molecules secreted by fungal cells perform several important functions, such as provision of nutrients, cell-to-cell communication, substrate colonization, detoxification of the environment and killing of potential competitors. Additionally, secretion of molecules by pathogenic fungi can be directly and/or indirectly involved in the molecular dialogue with the host, enabling their survival, multiplication and dissemination [152–154].

Published works have demonstrated that the *in vitro* treatment of *C. albicans* yeast cells with pepstatin A and some HIV PIs blocked the production and release of aspartic peptidases [110, 155, 156]. In order to exemplify this phenomenon, the incubation of *C. albicans* under nitrogen-limited conditions in the presence of indinavir, ritonavir or pepstatin A hampered the secretion of aspartic peptidases by approximately 90 % in relation to untreated cells, as judged through ELISA assay using an anti-peptidase serum [110]. Tipranavir and indinavir provoked in *C. albicans* a significant reduction on extracellular production of both peptidase and phospholipase [156], in which the latter represents a class of enzymes that cleaves phospholipids, the major components of biological membranes, resulting in lysis of host cells. Also, amprenavir diminished the secretion of esterase [111], a class of

hydrolytic enzyme that cleaves monoacylglycerol with a long chain fatty acid, aiding the host membrane disruption.

Corroborating the *in vitro* studies, in an elegant work conducted by De Bernardis et al. [157], highly active antiretroviral therapies (HAART) with HIV PI or non-nucleoside reverse-transcriptase inhibitors (NNRTI) were compared for their effect on prevalence, Sap production and the biotypes and antifungal sequential susceptibility of *Candida* spp. isolates from the oral cavity in a longitudinal prospective study. The results clearly showed that HAART-PI, but not HAART-NNRTI strongly inhibited Sap production in the oral cavity without exerting any consistent effect on the role of *Candida* spp. isolation or selection of low virulence or anti-mycotic resistant fungus biotype.

The treatment of *C. neoformans* yeast cells with indinavir for 24 h (25 μM) or 40 h (10 μM) induced a significant reduction on both the extracellular proteolytic activity and production of urease (which catalyzes the hydrolysis of urea to ammonia and carbamate and has been found to be an essential pathogenic factor for *C. neoformans*) in comparison to inhibitor-untreated cells. Contrarily, indinavir did not affect the production of secreted phospholipase and the pigment melanin [139]. Tipranavir also inhibited the secretion of peptidase and phospholipase, but did not interfere with both urease and melanin [156].

5.6.3 Surface Molecules

Surface molecules (e.g., polysaccharides, glycoproteins, glycolipids and enzymes) mediate interactions between fungal-fungal, fungal-host and fungal-environment, being critical to initial contact and the establishment of infection [23, 158]. Interestingly, the treatment of *C. albicans* yeasts with indinavir for 7 days induced a dose dependent decrease of cell-bound Sap antigen, as judged by flow cytometry analysis, where the inhibition increased from 27 % to 50 % as indinavir concentration increased from 0.05 mg/ml to 5 mg/ml [132]. Similar results were obtained with the treatment of yeast cells with amprenavir as judged by both flow cytometry and fluorescent microscopy [87]. Amprenavir also significantly reduced the exposition of mannose and sialic acid residues on the surface of *C. albicans* yeasts, which are carbohydrates constituent of numerous surface glycoconjugates directly linked to the yeast-adhesive processes, as well as diminished the amount of neutral lipids (mainly sterols) located within intracellular vesicles and at plasmatic membrane [111]. Indinavir (Fig. 12) and tipranavir were able to inhibit the polysaccharide capsular [156], which is considered the major virulence factor produced by *C. neoformans* [159].

5.6.4 Adhesion

Fungal adherence to an either biotic or abiotic surfaces is the critical first event in the establishment of an infection and some studies have showed the direct/indirect

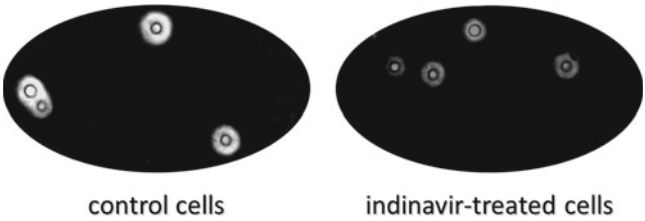

Fig. 12 Effect of indinavir on capsule size of *C. neoformans*. The yeast cells were cultured in the absence or in the presence of 10 **μ**M indinavir for 72 h. In order to evaluate capsule synthesis, *C. neoformans* was stained with India ink. The images of the most-representative capsule size-related events observed in untreated cells (very large capsules) and indinavir-treated cells (reduced capsules)

Table 6 Inhibition of the interaction between fungi and animal cells by aspartic PIs

Fungal species	Cell lineage	Aspartic PIs with effective action on the interaction process	References
C. albicans	Cervical cancer cells (HeLa)	Ritonavir	[162, 163]
	Human epidermal keratinocytes	Pepstatin A	[161]
	Human vaginal mucosa epithelial cells (VME)	Pepstatin A	[155]
	Kidney epithelial cells (Vero)	Ritonavir, saquinavir	[108]
	Reconstituted human epithelium (RHE)	Saquinavir, indinavir	[109]
C. neoformans	Peripheral blood mononuclear cells (PBMC), polymorphonuclear leukocytes (PMNL)	Indinavir, tipranavir	[139, 156]
	Murine microglial cells (BV2)	Indinavir	[115]
P. jiroveci	Human lung fibroblast cells (HEL299)	Tipranavir, amprenavir, indinavir, ritonavir, nelfinavir, saquinavir	[140, 141, 144]
F. pedrosoi	Chinese hamster ovary cells (CHO), mouse macrophages (RAW), mouse fibroblasts (3T3-L1)	Indinavir, ritonavir, nelfinavir, saquinavir	[107]

involvement of aspartic peptidases in the attachment with host tissues/cells and maintenance of fungal virulence [101–104]. So, the removal of these surface structures expressively alters the fungal adherence behavior.

Overall, the reduction expression (exposition/production) of surface aspartic peptidases (e.g., Saps in *C. albicans*), glycoconjugates rich in mannose and/or sialic acid residues, polysaccharides (e.g., capsule in *C. neoformans*) and membrane sterols due to the treatment with aspartic PIs culminates in the inability of fungi in adhering to abiotic substrates (e.g., polystyrene and acrylic) [87, 111, 160] as well as to different cell lineages [87, 107–109, 140–145, 155, 161–164] (Table 6 and Fig. 13). The inhibition of binding to inert substrates leads to the incapability to form biofilms. Biofilm poses a number of clinical challenges due to their resistance to both immune defense mechanisms and antimicrobials [165, 166].

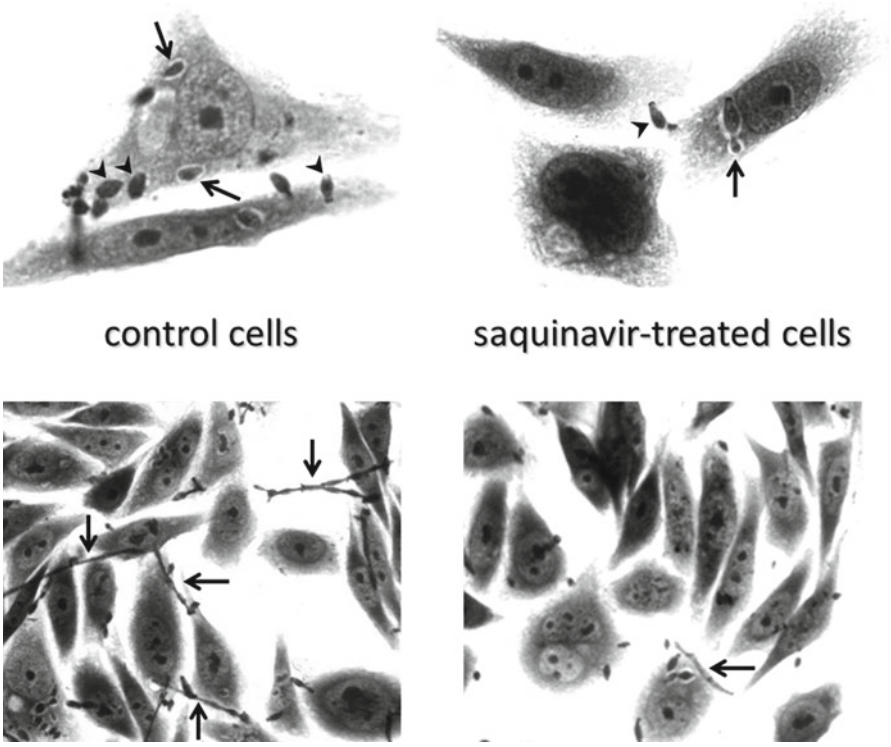

Fig. 13 Optical microscopy showing the representative images of the interaction between *F. pedrosoi* conidia and Chinese hamster ovary (CHO) cells. The conidial cells were pre-treated for 1 h in the absence or in the presence of saquinavir at 100 **μ**M, washed and then interacted with CHO cells for 2 h (*upper images*) and 4 h (*lower images*). Note in the superior microscopies that conidial cells were able to adhere (*arrowheads*) and invade (*arrows*) the epithelial cells; however, these processes were drastically diminished after the treatment with saquinavir. Addendum: ingested conidia are located within vacuole. Note in the inferior panels that in PI-untreated fungi, some conidial cells transform in hyphae (*arrows*), showing the well-known differentiation process triggered by the contact of conidia with host cells. Contrarily, when conidia were pre-treated with saquinavir, the number of hyphae is drastically reduced

5.7 In Vivo *Experimental Fungal Infection*

The aspartic PIs have been tested in animal experimentation to determine their effectiveness at attenuating fungal infections, especially against *C. albicans* and *C. neoformans*. Regarding *C. albicans*, pepstatin A showed a protective effect in murine and rat mucosal models [167, 168]; however, failed to protect murine disseminated-infection [169–172]. Pepstatin-like drugs, however, are not used clinically because of their metabolism in the liver and rapid clearance from blood [171]. Ritonavir and indinavir also demonstrated therapeutic effect in a rat vaginitis model [110].

In *C. neoformans*, tipranavir showed a therapeutic effect in experimental systemic cryptococcosis, as evaluated by reduced fungal burden in brain and liver of immunocompetent and immunodepressed mice [156].

6 Conclusions

The current therapeutic arsenal against fungi is clearly inadequate and underscores the urgent need to develop new effective, safe and cost-effective drugs. Due to multifaceted functions, aspartic peptidases are genuine target for driving alternative chemotherapeutical compounds against human fungal pathogens. As summarized herein, *in vitro* and *in vivo* well-elaborated works clearly have demonstrated that aspartic PIs were capable in blocking vital events of both yeasts and moulds, generating several disturbances in cellular organization that culminate in fungal death. The authors really hope that all these findings together arouse the curiosity and the enthusiasm of other researchers in order to look for novel compounds with the ability to inhibit aspartic peptidases produced by human fungal pathogens. The novel compounds must be more specific, powerful and with reduced side effects, in an attempt to increase our armamentarium to treat fungal diseases.

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Cysteine Proteases of Human Malaria Parasites

Kailash C. Pandey

Abstract There is an urgent need for new drugs against malaria, one of the most important infections of human which takes millions of lives annually. Cysteine protease inhibitors have demonstrated anti-malarial effects, and they are potential new drug targets, especially when current drugs are showing resistance. Falcipains and vivapains are best characterized cysteine proteases of *P. falciparum* and *P. vivax*, respectively. Using cysteine protease inhibitors and manipulating cysteine proteases specific genes have confirmed their roles in hemoglobin hydrolysis. In *P. falciparum*, falcipain-2 and falcipain-3 are major hemoglobinases that hydrolyzes human hemoglobin. Vivapain-2, vivapain-3 and vivapain-4 are important cysteine proteases of *P. vivax*, which shared a number of features with falcipain-2 and falcipain-3. Structural and biochemical analysis of falcipains and vivapains showed that they have specific domains for specific functions. These include trafficking domain, inhibitory domain, refolding domain and hemoglobin binding domain. Recent study also indicates the mechanism of auto-activation of falcipains, where salt bridges and hydrogen bonds between pro-mature domains play crucial role. Study indicates that cysteine and aspartic proteases work collaboratively to enhance the parasites' ability to hydrolyze host erythrocyte hemoglobin. Recent advances in cysteine proteases biochemistry and the complexes of cysteine proteases with small and macromolecular inhibitors provide structural insight to facilitate the drug design. Therefore, giving due importance to the cysteine proteases, this chapter will focus the recent advancement in the field of cysteine proteases of human malaria parasite and prospects for exploitation as drug targets.

Keywords Hemoglobinases • Drug resistance • Protein–protein interactions • Hot-spot • Hemoglobin binding domain • Refolding domain • Anti-malarial

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1 Introduction

Evidence begin in the middle of the 1800s, Pasteur, Kotch and his co-workers had provided convincing evidence that microbes can cause disease, and identified disease causing agents, called parasites. The understanding of the malaria parasites begins in 1880 with the discovery of the parasites in the blood of malaria patients by Alphonse Laveran. Later, it was Ronald Ross, a surgeon in the Indian Medical Service, who showed that malaria was transmitted by the bite of an infected female mosquito [1]. After these land mark discoveries, there are lots of advancement to our understanding of the integrated interrelationship between malaria parasite and their host as well as parasite biology. Recently researchers developed a highly sensitive and quantitative detection of malaria based on parasite topoisomerase 1 [2]. Importantly, advances in malaria biochemistry open new light in the field, provided important drugs and vaccine targets and help us to develop better diagnostics and mechanism based approach for anti-malarial chemotherapies. Malarial structural biology and biochemistry becomes the major sources for providing potential targets based on structure-function. *P. falciparum* and *P. vivax* are the most predominant human malaria species worldwide. Recent global malaria mortality data show that malaria is the underlying cause of death for 1.24 million individuals [3]. Although other human malaria parasite, *P. vivax* is less virulent than *P. falciparum*, but it is substantially distributed and causes extensive morbidity. Together, these two malarial parasites are responsible for ~90 % of episodes of human malaria [4]. The RTS, S vaccine that targets the circumsporozoite protein has shown 50 % protection against *P. falciparum* malaria [5]. The big problem is the complexity of malaria parasites, and has a multiphase life cycle that means it shows a different side to the immune system at different stages of its development. Malaria parasite has more than ~5,000 genes, which are ~50 times more than viruses. There is a big challenge to immunologist because it is difficult to see which of the tsunami of immune responses give rise by the parasite. A number of drugs are currently available to treat malaria [6], however, treatment is becoming complicated by drug resistance. Drug resistance against the new effective drug, Artemisinin, is also emerging [7–9]. To develops effective compounds, it is very important to characterize the structural and biochemical features of new drug targets. Among potential new targets for antimalarial chemotherapy are *Plasmodium* proteases. Proteases are druggable targets, and at present protease inhibitors are now licensed as well as in clinical development to treat different diseases. This chapter will survey recent available information on falcipains and vivapains, cysteine proteases from *P. falciparum* and *P. vivax*, respectively.

2 Malarial Protease Nomenclature

Cysteine poetesses utilize a catalytic cysteine that mediates protein hydrolysis via nucleophilic attack on the carbonyl carbon of a peptide bond. Cysteine proteases are divided into different clans, and further subdivided into families based on sequence

identities and similarities [10]. Clan CA, family C1 proteases use catalytic Cys, His, Asn residues at the active site. Family C1 comes under papain family cysteine proteases. The term "falcipain" comes from falciparum (falci) and papain (pain). This chapter is deal with two major papain family cysteine proteases in *P. falciparum* (falcipain-1, falcipain-2, falcipain-2′ and falcipain-3) and *P. vivax* (vivapain-2, vivapain-3, vivapain-4).

3 Expression of Proteases by Erythrocytic Parasites

Using immunoelectron microscopy and labeling with specific antibodies, falcipain-2 and falcipain-3 differ in their timing of expression. Falcipain-2 is expressed about 12 h earlier than falcipain-3. Falcipain-2 is predominant express in early and late trophozoites, whereas, falcipain-3 can be seen in late trophozoites and early schizonts [11, 12]. Immunoblots have identified the expression of falcipain-1 across the erythrocytic cycle [13]. However, proteomic analysis suggested that it express only in sporozoites [14]. The localization for vivapains has been highly limited not only because of low parasitemia in the infected blood sample, but also due to lack of in vitro culture of *P. vivax*. Immunoblots have identified the expression of vivapain-4 in all intra erythrocytic stages of *P. vivax* [15, 16]. The majority of vivapain-4 localization appeared to be limited to the food vacuole with dark hemozoin pigment, while some protein was also labeled diffusely in the parasite cytoplasm [16].

4 Cysteine Proteases as Hemoglobinases

Brown (1911) first suggested that the parasite degrade hemoglobin by the action of proteolytic enzymes to produce malaria pigment, but it was Moulder and Evan (1946) found such an enzyme known as acid protease, in extracts of *P. gallinaceum* [17, 18]. Now in advance age of biochemistry, it has been well established that parasite has different class of proteases; aspartic, serine, metalloproteases, and importantly, cysteine proteases are very well studied as hemoglobinases. The effects of E-64 and leupeptin (major cysteine protease inhibitors) against cultured *P. falciparum* parasites suggested that cysteine proteases are major hemoglobinases (Fig. 1) [19]. Parasite treated with cysteine protease inhibitor showed dark food vacuole duo to accumulation of undegraded hemoglobin (Fig. 1). Disruption of falcipain-2 gene results in reduced hemoglobin hydrolysis in the trophozoite stage and accumulation of undegraded hemoglobin in the parasite food vacuole [21]. However, disruption of falcipain-3 could not be achieved, but the gene was readily replaced with a tagged functional copy, indicating that falcipain-3 is essential for erythrocytic parasites [22]. It has been suggested that hemoglobin hydrolysis by falcipains are not a highly ordered process, but rather proceeds with rapid cleavage by enzymes at multiple sites [23]. A knockout of falcipain-1 and falcipain-2′ had no obvious phenotype, but the

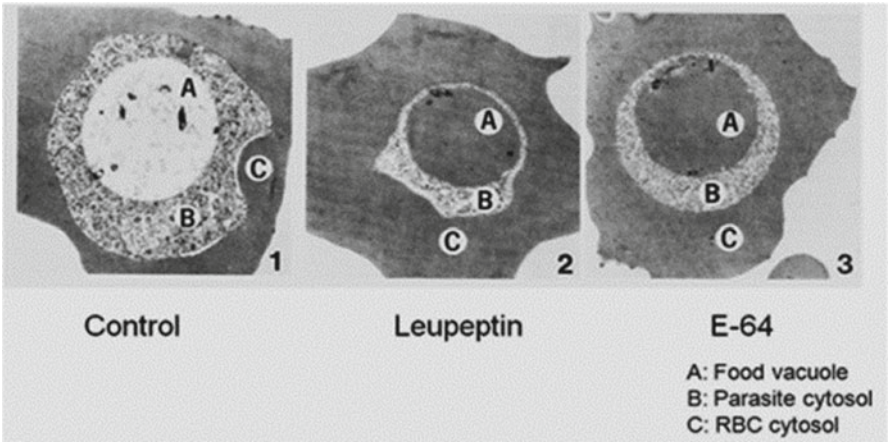

Fig. 1 Cysteine protease inhibitors inhibit hemoglobin hydrolysis; Electron microscopy of infected erythrocytes with *P. falciparum*. Parasites treated with leupeptin and E-64, showed dark food vacuole (Rosenthal PJ, Curr Pharm. Des. 2002 and Pandey KC, J Parasitic. Dis. 2011)

disruption of the falcipains-1 gene led to decrease production of oocytsts in mosquitoes [13, 24]. Altogether the available data show that falcipain-2 and falcipain-3 play key roles in hemoglobin degradation by erythrocytic parasites [11, 12, 21].

In case of *P. vivax*, vivapain-2, vivapain-3 and vivapain-4, are also described as major hemoglobinases [15, 16]. Biochemical analysis showed that like falcipains, vivapains also act as hemoglobinases. Recent study indicated that plasmepsin-4, an aspartic protease of *P. vivax*, acted synergistically with vivapain-2 and vivapain-3, in the hydrolysis of hemoglobin [25]. This result suggests that plasmepsin-4 and vivapains works collaboratively to enhance the parasites' ability to degrade hemoglobin.

5 Cysteine Proteases Involved in Erythrocyte Rupture

Cysteine proteases inhibitors block the rupture of red blood cells. Therefore, it is believed that cysteine protease activity is required for the release of merozoites. Older studies have shown that the accumulation of mature schizonts in *P. falciparum* cultures treated with leupeptin [26]. Further, Salmon et al. have shown that the E-64 blocked lysis of the schizont parasitophorous vacuole membrane. These studies have suggested that cysteine protease activity is requires for the hydrolysis of parasitophorous vacuole membrane associated proteins to mediate merozoite released [27]. In another study using antipain and leupeptin blocked lysis of the erythrocyte membrane [28]. Based on these results it appears that the release of merozoites in a two steps process, one is hydrolysis of proteins associated with the

parasitophorous vacuole and other is hydrolysis of erythrocyte membranes [28]. Although the sequence of these two steps is still unknown. Falcipain-2 has been shown to hydrolyze the red blood cell cytoskeletal proteins, band 4.1 and ankyrin. Similarly, vivapain-2 and vivapain-3 also have shown to hydrolyze spectrin, protein 3, actin, glycophorin A and protein 4.1 [15, 29, 30].

6 Function of Different Domains of Malarial Proteases

6.1 The N-terminal Part of the Prodomain Is Required for Trafficking of Falcipains to the Food Vacuole

Falcipains and vivapains have two main domains, the prodomain and the mature domain. These domains further divided into small sub domains (Fig. 2a). Using transfection technology and constructing different chimeras, it has been shown that the prodomain of falcipain-2 and falcipain-3 were sufficient to target active enzyme to the food vacuole. Serial and truncations studies showed that 20 amino acid of luminal portion and a 10 amino acid stretch of the cytoplasmic portion of N-terminal of the prodomain were essential for food vacuole trafficking of falcipain-2 and falcipain-3 [31]. These result indicated that falcipains utilized bipartite motif dependent mechanism for targeting to the food vacuole through the route of plasma membrane and cytostomal vesicles [31].

6.2 The Prodomain-Mature Domain Interactions Are Necessary for the Auto-Activation of Falcipain-2 and Falcipain-3

Our previous studies demonstrated that these enzymes are equipped with specific domains for specific functions (Fig. 2a, b) [32, 33]. As with many proteases, falcipain-2 and falcipain-3 are synthesized as inactive zymogens. Both enzymes are synthesized as membrane bound pro forms that are processed to soluble mature forms. Study revealed that prodomain-mature domain of falcipain-2 and falcipain-3 interacts via salt bridges and hydrophobic interactions [34]. Mutagenesis result showed that two salt bridges (Arg^{185} - Glu^{221}, Glu^{210} - Lys^{403}) in falcipain-2, and one salt bridge (Arg^{202} - Glu^{238}) in falcipain-3, play crucial roles in the activation of these enzymes. Further study revealed that hydrophobic interactions present both in falcipain-2 (Phe^{214}, $Trp^{449}Trp^{453}$) and falcipain-3 ($Phe^{231}Trp^{457}Trp^{461}$) also play important roles in the activation of these enzymes. Based on mutagenesis study, we proposed a model that explains the prodomain and the mature domain interactions are necessary (Fig. 2c).

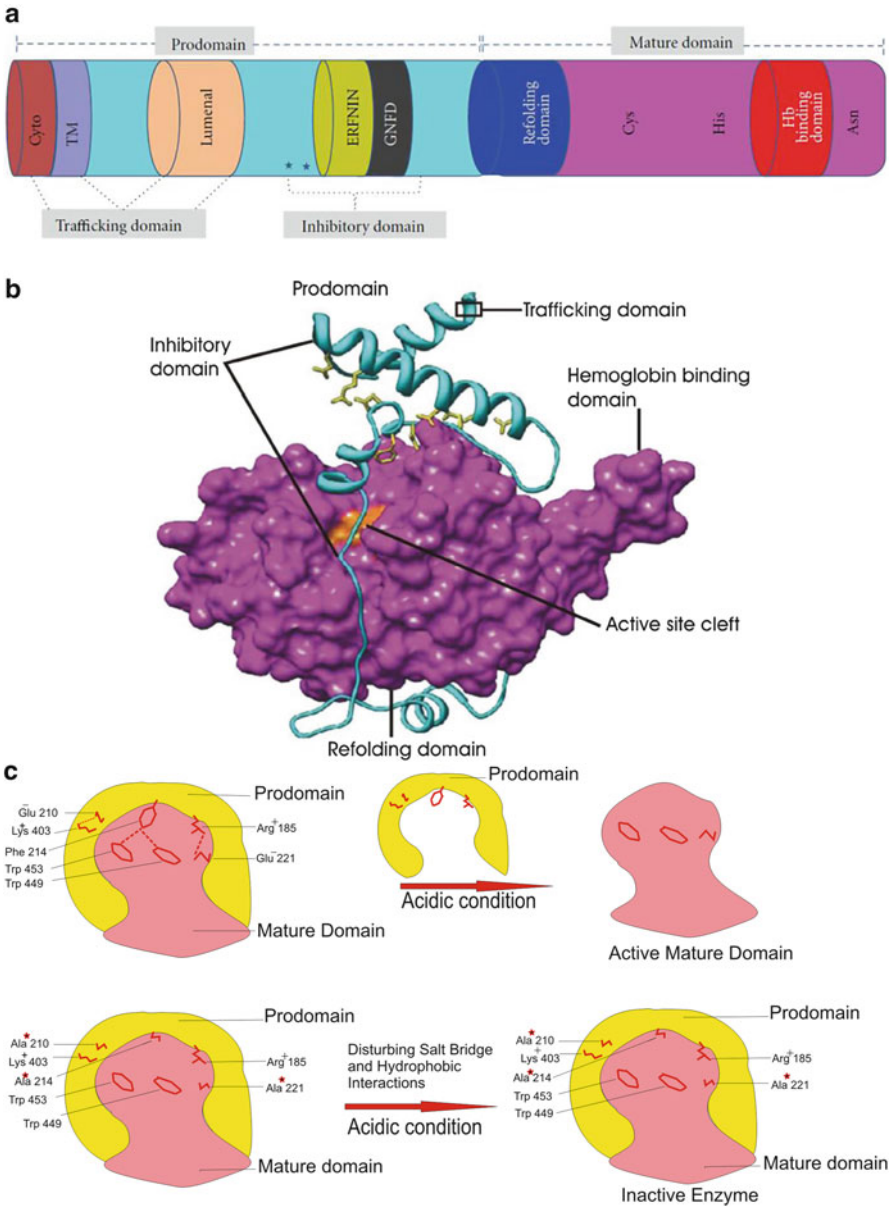

Fig. 2 (**a**) Domains of falcipain 2: The prodomain is made up of cytoplasmic, transmembrane, luminal, which contribute to targeting of enzyme, and C-terminal of the prodomain has inhibitory domain. The mature domain has refolding domain, hemoglobin (Hb) binding domain, and catalytic

6.3 The C-terminal Part of the Prodomain Is Required for Inhibition of the Mature Domain

Like many other proteases, falcipains and vivapains are synthesized as a zymogen, and the prodomain inhibits the activity of the mature enzyme [11, 15, 35]. It has been found that a C-terminal segment (Leu^{155}–Asp^{243}) of the prodomain, including two motifs (ERFNIN and GNFD) that are also conserved in cathepsin L subfamily proteases, mediates prodomain inhibitory activity (Fig. 2a). Earlier work with other cathepsin L subfamily proteases suggests key roles for conserved hydrophobic amino acids (phenylalanine and tryptophan) as well as the ERFNIN and GNFD motifs in maintaining the prodomain structure [36]. Secondary structure was seen in a fragment with potent inhibitory activity (Leu^{155}–Asp^{243}), but not in two larger constructs that lacked any sequence downstream of the ERFNIN and GNFD motifs or in a peptide spanning the ERFNIN and GNFD motifs [36]. These results indicate that together with ERFNIN and GNFD motifs, an upstream region including two conserved Phe residues are required for proper folding or maintenance of secondary structure of the prodomain which is required for inhibition (Fig. 2a, b).

6.4 The N-terminal Part of the Mature Domain Is Required for Refolding

Falcipain and vivapain subfamily proteases have features that are unusual for family C1A proteases. They encode short N-terminal extensions of the mature domain, which is unique among described papain family proteases [35]. The N-terminal extensions allow folding of the mature domain to active enzymes [37]. Other papain family enzymes required the prodomain for proper refolding [35]. The Only 12 amino acids of the N-terminus extension of which can mediate correct folding either when it is included upstream of the catalytic domain or when the catalytic domain was refolded with a separate prodomain-folding domain construct but not with an isolated folding domain peptide [35, 37] (Fig. 3). These results indicated that the prodomain mediates the interaction between the mature and folding domain when

Fig. 2 (continued) triad residues (Cys, His, Asn). The conserved residues (Phe^{165}, Phe^{168}) present in inhibitory domain are labeled with asterisks (Pandey KC, et al., 2012. Journal of Tropical Medicine). (**b**) Structure of Pro-falcipain 2: The mature domain and the prodomain are showing in purple and cyan, respectively (Wang et al., 2006; PNAS; Pandey et al., 2009 PLoS One). Different domains are labeled and catalytic triad residues in orange. (**c**) Proposed model of activation. Model showing mechanism of activation of falcipain. The prodomain (*yellow*) and the mature domain (*pink*) are stabilized by salt and hydrophobic interactions (shown by *red dashes*). The wild enzyme (*upper panel*) processed normally in acidic condition whereas, mutants (mutations indicated by *red* asterisks) with disrupted salt and hydrophobic interactions failed to process into active enzyme (Srinivasan et al., 2012, PLoS One)

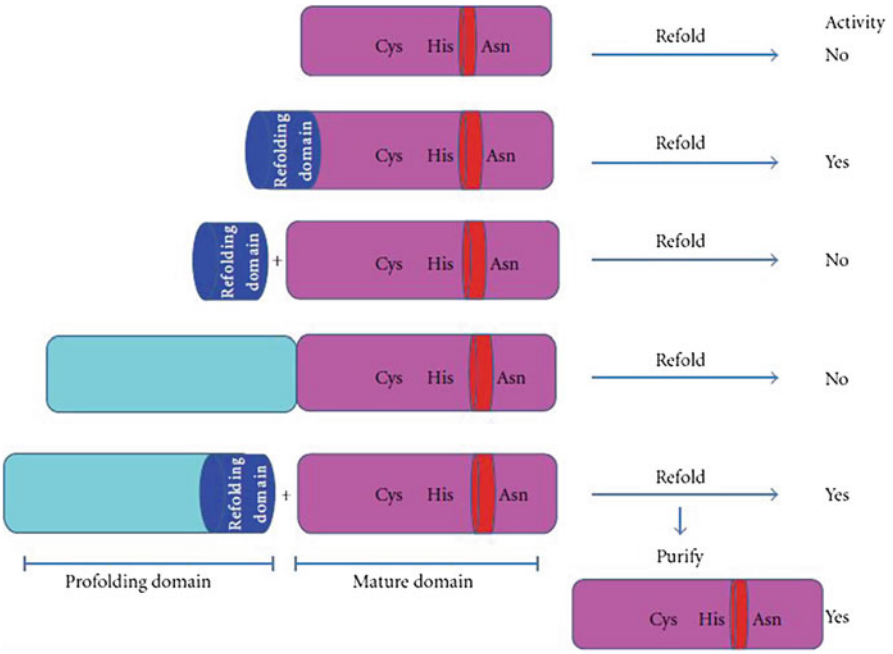

Fig. 3 Refolding of enzyme. Different constructs were used to study refolding. The mature domain was expressed alone or with the refolding domain either in cis or trans and the activity was assayed as described in figure (Pandey KC, et al., 2012. Journal of Tropical Medicine)

they were not covalently bound. Further, it been shown that refolding domain only required for refolding not for activity (Fig. 3) [37]. The N-terminal part is functionally conserved within the falcipain-2, falcipain-3, vivapain-2, vivapain-3 and vivapain-4 [37]. It has been showed that refolding domain can be swapped between the plasmodial proteases that harbor the same function. Structural analysis further reveals that the folding domain and the mature domain are stabilize by a hydrogen bond and a salt bridge [38].

6.5 The C-terminus Insert of the Mature Domain as a Hemoglobin Binding Domain

Falcipains also have an insertion near C-terminal of the mature domain that is unusual for papain family proteases (Fig. 2a, b) [39]. A motif of identical size (10 aa) is found in all studied proteases of this subfamily including falcipains-2 and falcipain-3 and vivapains, but not present in falcipains-1. The secondary structure in this region is well conserved between falcipain-2, falcipain-3 and vivapain [39]. Removal of this 10 aa insert had no effect on activity of falcipains-2 against a number of peptide and protein substrates. In contrast, the mutated protease had loss in activity

against hemoglobin. These result stated that loss in activity was due to an inability of the mutant protease to bind hemoglobin [39]. The binding affinity (kDa is 3.3 μM) between falcipains-2 and hemoglobin had been demonstrated by Biocore [40]. These data indicated that falcipain-2 capture hemoglobin via its C-terminus motif.

7 Structural Basis Inhibition of Falcipains and Vivapains

7.1 Inhibition by Macromolecules

7.1.1 Inhibition of Falcipain by Cystatin

There are two major classes of cysteine protease inhibitors, small inhibitors like leupeptin, vinyl sulfones, E-64, and another class known as macromolecular inhibitors. Macromolecule inhibitors are polypeptide in nature, which are generally present inside the organisms. These endogenous cysteine protease inhibitors have been described in a number of eukaryotic systems. Here, we will discuss three major cysteine protease inhibitors, cystatin, chagasin, and falstatin. Wang et al. studied the falcipain-cystatin interactions by solving the structure of this complex [38]. Cystatin was used as a falcipains active site inhibitor with the expectation that protein-protein interactions over large surfaces would enhance crystal quality and thus facilitate structural determination. Cystatin inhibit a wide range of papain family cysteine proteases with high affinity making them ideal candidates for co crystallization with falcipains and vivapains. Falcipain and cystatin formed 1:1 complex (Fig. 4a). Similarly it was predicted for vivapains. The inhibitory constants (Ki) of cystatin for falcipain-2 and falcipain-3 are 6.5 nM and 100 nM, respectively [38]. Cystatin is a more potent inhibitor of falcipain-2 than falcipain-3, which suggests that cystatin regulates both the falcipains with different rates. This might be important biologically, since their timing of expression is slightly different. Cystatin largely interacts with falcipain-2 at prime sites where substrate-binding pockets are relatively shallow and less defined [38].

7.1.2 Inhibition of Falcipain by Chagasin

Chagasin is a cysteine protease inhibitor that was first identified in *T. cruzi* as the physiological regulator of cruzain, the major cysteine protease [41]. Chagasin is also a potent inhibitor of falcipains and vivapains demonstrates 1:1 binding. The protease-binding loops (BC, DE, and FG) in chagasin form a well-aligned wedge that fills the active site groove of falcipain-2 to obstruct substrate binding [41]. The BC loop is one of the three signature motifs that contribute to the inhibition of the cysteine protease. It is important to note that Thr 31 in the BC loop of chagasin binds to the catalytic Cys at the falcipain-2 active site by water-mediated hydrogen bonds [41]. Another feature of the falcipain-2-chagasin interaction is the highly mobile DE loop like in E-64, a strong irreversible inhibitor of cysteine protease, which occupies the nonprime site. In summary, mostly BC loop contribute to inhibit the active site of enzyme.

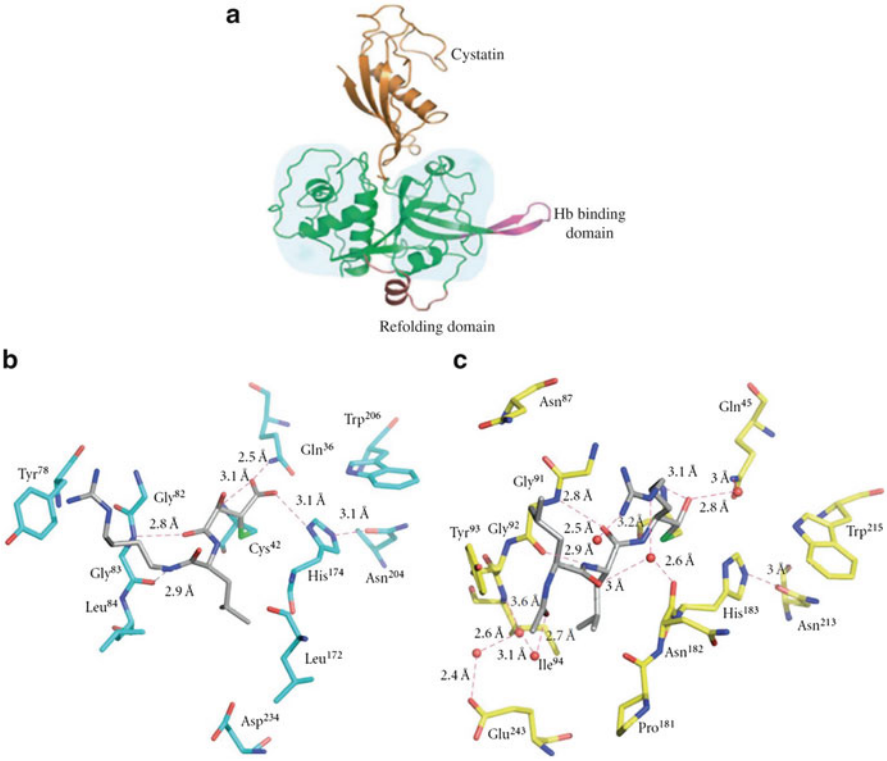

Fig. 4 (**a**) Structure of falcipain 2-cystatin complex: Cystatin is colored in *orange*, and falcipain-2 protease is colored in *green*. Refolding domain and hemoglobin binding domain are highlighted in *pink* and *salmon*, respectively (Wang et al., 2006, PNAS). (**b**) Interaction between falcipain 2 and E-64. The residues in the active site of falcipain-2 are colored *blue* and labeled, and E-64 is colored in *grey*. The interactions with enzyme and inhibitor are in *pink* (Kerr et al., 2009, JMC). (**c**) Interaction between falcipain 3-leupeptin: The residues in the active site of falcipain-3 are colored *yellow* and labeled, and leupeptin is colored in *gray*. The interactions with enzyme and inhibitor are in *pink* (Kerr et al., 2009, JMC)

7.1.3 Inhibition of Falcipain and Vivapain by Falstatin

Falstatin has been recognized as an endogenous cysteine protease inhibitor in *P. falciparum*. The homologue of falstatin has been found in *P. vivax, P. berghei and P. yoelii*. Erythrocytic *P. falciparum* parasites express falstatin in early ring and schizont, but not in trophozoites, the stage at which the cysteine protease activity of *P. falciparum* is maximal [42]. The stage-specificity of falstatin expression and the effects of anti-falstatin antibodies on parasite development suggest that this inhibitor facilitates invasion of erythrocytes. Falstatin is a competitive and reversible inhibitor of falcipains and vivapains, as demonstrated by increasing calculated *Km* values but similar *V*max values with increasing concentrations of falstatin, and demonstrate 1:1 binding [41]. Falstatin inhibit falcipains and vivapains with almost similar Ki [41]. But it is unknown how falstatin regulate the *P. falciparum* cysteine proteases. Our recent data suggest

that hydrophobic interaction and hydrogen bond within the BC loop of falstatin are required for cysteine protease inhibition of falcipains-3 and vivapains-2 (Shrinivasan et al., 2013 pers. comm). It seems *P. falciparum* and *P. vivax* and other homologues express an endogenous cysteine protease inhibitor presumably for control of parasite or host proteases activity.

8 Drug Based on Protein–Protein Interactions (PPIs), a New Approach

PPIs targets have deliberately been avoided by small molecule drug developers, largely because of technological hurdles. Recent scientific advancement, however, suggest that these challenges are now becoming more achievable [43]. Evidences have demonstrated that small molecules can disrupt the large and complex protein interactions by binding to interface called "hotspots" [44]. Recently, Hyunil et al., have designed a highly specific inhibitor of calpain by mimicking a natural protein-protein interaction between calpain and its endogenous inhibitor calpastatin [45]. Further, researchers target to block the interactions of two monomers, and prevent it forming the active dimer interface, and developed the first small-molecule inhibitor of a herpes virus protease that block PPIs [46]. Recently our group is also focusing on protein interactions crucial for activation of enzyme [34]. Other target is C-terminal insert of falcipain-2 and falcipain-3, which specifically bind to hemoglobin, a natural substrate of falcipains and vivapains [39]. Since, C-terminal motif of falcipain, act as a hemoglobin binding domain, and the disruption of C-terminal motif-hemoglobin interaction should offer a novel means of inhibition of parasite development.

Disturbing the protein-protein interactions, one can think to get rid of the key to a specific lock. In fact, by blocking protein–protein interaction, we don't need the key to a specific lock. A drug design view point targeting to protein-protein interactions, which is opposed to an enzyme active site could be interesting field in drug discovery. Targeting "hot-spot" in protein-protein interactions could be less prone to drug resistant mutation. This is because acquiring a drug resistance point mutation may not be companied by the right compensatory mutations, which is less likely to be tolerated by a complex protein-protein interface.

9 Inhibition of Malarial Cysteine Proteases by Small Molecule

Leupeptin and E-64, are major cysteine protease inhibitors that bind to the active site [46, 47]. The structures of falcipain-2 and falcipain-3 have been determined in complex with these small inhibitors. E-64, and leupeptin interact with residues in the S1, S2, and S3 sub sites of falcipain-2 and falcipain-3, corresponding to the P1, and P2, and P3 position of the inhibitors (Fig. 4b, c). The conserved catalytic residues of falcipain-2 and falcipain-3 ($Gln^{36/45}$, $Cys^{42/51}$, $His^{174/183}$, $Asn^{204/213}$,

respectively) are similarly oriented with respect to the co-crystallized inhibitors. Inhibitors display binding modes with their partner enzymes similar to those found in other papain family enzymes [47, 48]. In the case of the falcipain-2 complex with E-64, enzyme active sites Gln^{36}, Ser^{41}, Cys^{42}, Asn^{81}, and His^{174} are involved in the formation of additional hydrogen bonds with E-64 (Fig. 4b). In the falcipain-3-leupeptin complex, Gln^{45}, Cys^{51}, and Asn^{182} also act as hydrogen bonding partners to the inhibitor. The enzyme-inhibitor complex stabilize by a series of possible hydrophobic interaction using nonpolar region of Gly^{40},Tyr^{78}, Gly^{82}, leu^{84}, Ser^{149}, Leu^{172}, Asn^{173}, and Ala^{175} in falcipain-2, and $Tyro^{90}$, Gly^{91}, Tyr^{93}, Ile^{94}, and Ser^{158} in case of falcipain-3 (Fig. 4c). The active site cysteine of falcipain-3 forms a covalent, reversible hemithioketal with the asymmetric carbonyl carbon of leupeptin [46]. The effect of leupeptin and E-64 on vivapain-2, vivapain-3, indicated that the patterns and sensitivities are similar with falcipains. Notably, vivapain-2 was much more sensitive to E-64 than were falcipain-2 and falcipain-3 [15]. There are potential cysteine proteases inhibitors as anti-malarial e.g. peptidylfluromethyl ketone, vinyl sulfones, and aldehyde [47, 48]. *P. falciparum* mouse model was recently used to show potent in vivo anti-malarial activity of falcipain inhibitors [49].

10 Conclusion

A number of studies have supported the concept with the demonstration that cysteine proteases inhibitors have potent in vitro and in vivo anti-malarial effect. In recent year, our understanding of the cysteine proteases repertoire of malaria parasites has noticeably increased by advancement of biochemistry and structural biology. The detail structure-function study of these enzymes and interaction with inhibitors will provide insight to develop new drug against malaria. Structure-function guided approaches should have great role in the design of potent and highly selective inhibitor. Efforts to optimized new inhibitors based on the structure-function of these cysteine proteases are currently underway. I hope that this current overview of malarial cysteine proteases will updated the field, and inspire others to contribute their talents and expertise to achieve success in one of the world's most deadly diseases.

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Proteases as Virulence Factors in *Leishmania*: Focus on Serine Proteases as Possible Therapeutic Targets

Partha Das, MD Nur Alam, Tripti De, and Tapati Chakraborti

Abstract Leishmaniasis is one of the most assorted and intricate of all vector borne diseases caused by the genus *Leishmania*. Survival of *Leishmania* parasites inside the mammalian host needs a set of virulence factors, among them, *Leishmania* proteases have paramount importance. Several of these proteases have been identified as potential virulence factors for their crucial roles in the invasion of the host via parasite migration through tissue barriers, degradation of host proteins for nutrition purpose, immune evasion and activation of inflammation. Hence, the investigation on proteases in *Leishmania* is proposed as a valuable approach to enhance our knowledge on host-parasite interaction. Through various studies, a number of metalloproteases and cysteine proteases have been implicated as major components in host invasion by modulating host cell signaling for the establishment and continuation of infection by *Leishmania*. But, the roles of serine proteases in leishmaniasis have not been investigated adequately. In this review, we will discuss the significance of *Leishmania* proteases in parasite lifecycle and their possible accountability as a new drug target with special emphasis on *Leishmania* serine proteases.

Keywords Leishmaniasis • Matrix metalloproteases • Oligopeptidase B • Cysteine protease • Serine protease • Virulence • Protease inhibitor • Drug target • Chemotherapy

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1 Introduction

Leishmaniasis is caused by an obligate intracellular protozoan parasite of the genus *Leishmania*. The disease is manifested in different clinical forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) of which VL is the most severe form of leishmaniasis. According to World Health Organization (WHO), 350 million people are at risk of contracting one of the forms of the disease, 12 million cases in 88 endemic countries worldwide and also with an annual-incidence of 1.5 million new cases [1]. Currently, leishmaniasis is spreading due to co-infection with human immunodeficiency virus (HIV) [2].

All types of leishmaniasis are transmitted by the female phlebotomine sand flies. *Leishmania* parasites alternate between two distinct developmental stages. The motile flagellated promastigote forms multiply and develop extracellularly in the alimentary tract of the blood sucking female sand fly vectors and are transmitted during the blood meal into mammalian host. Inside the mammalian hosts promastigotes infect macrophages of the reticuloendothelial tissue and differentiate into nonmotile amastigotes forms which multiply as such in the phagolysosomal vacuoles.

Like other intracellular protozoan parasites, *Leishmania* survive inside the host macrophages (Mφs) by implying various mechanisms [3, 4] and allow them to evade and suppress the host immune system, thereby granting their survival in the host body. In order to establish themselves in the host, *Leishmania* promastigotes have to escape Mφ microbicidal action and amastigotes have to repress Mø killing abilities to re-invade new Mφs for persistent *Leishmania* infection [5, 6]. Following phagocytosis, *Leishmania* persuade the harsh environment through the inhibition of hydrolytic enzymes, toxic metabolic products, cell signaling, cytokine production and other events [7]. These tactics permit *Leishmania* to successfully undermine the host innate and acquired immune responses to promote their survival. The effective elimination of parasites by macrophages depends on the activation of appropriate immune responses [8, 9]. So, *Leishmania* have developed mechanisms to subvert the microbicidal activity of Mφs [10] where the host Mφs produce nitric oxide (NO) through the induction of iNOS, in response to extracellular signals, including IFN-γ and LPS [11] and this NO generation by activated Mφs is the prerequisite for intracellular killing of amastigotes. *Leishmania* enter into the host Mφs by receptor mediated endocytosis and avoid complement mediated lysis by cleaving C_{3b} to C_{3bi} [7]. Inside the phagolysosomes of the host Mφs, *Leishmania* promastigotes can also hinder phagosome–endosome fusion and protect themselves from toxic oxygen metabolites generated during the macrophage oxidative burst by scavenging hydroxyl radicals and superoxide anions [12]. *Leishmania* also escape microbicidal action of host Mφs by modulating host cell cytokines production, where induction of a Th1 type immune response is associated with clearance of *Leishmania* infection and a Th2 type immune response leads to persistence of infection. In *Leishmania* infection TGFβ, IL-10 and PGE2 have been implicated as important immunosuppressive signaling molecules and also inhibit the production of the Th1 response-promoting cytokines IL-1, IL-12 and TNF-α [13, 14].

Both promastigotes and amastigotes alter the signaling pathways of Mφs in order to block their killing functions [14–17]. PKC signaling is known to play a key role in the regulation of Mφ functions activating Th1 response leading to NO production and oxidative burst [14] but, restraining of PKC activation by *Leishmania* parasites impair subsequent signaling phenomena [14, 15].

Leishmania modify host signaling through the disruption of cellular phosphorylation [18] by expressing endogenous phosphatases that cause a decrease of macrophage PKC activity and inhibition of MKP1 (p38) and MMKP3/PP2 (ERK1/2) activation leading to the up regulation of IL-10 and down regulation of NO and TNF-α production [14]. Consequently, *Leishmania* mediated IFN-γ inducible tyrosine phosphorylation and activation of JAK1, JAK2, and STAT1 pathway [19] has been shown to involve the activation of the cellular protein tyrosine phosphatase SHP-1, responsible for dephosphorylating MAP kinases 1 and 2 [14, 15]. The *Leishmania* parasites can also decline the nuclear translocation of NF-κB in monocytes with an outcome of a decrease in IL-12 production [20].

Various virulence factors enable *Leishmania* to invade and establish infection inside the mammalian host [7, 14, 21–25], these factors include major surface protease (GP63), cysteine proteases (CPs), serine proteases (SPs), lipophosphoglycan (LPG), A2 protein family, glycosylinositol phospholipids (GIPLs), secreted acid phosphatases (SAPs) and kinetoplastid membrane protein 11 (KMP-11) etc. However, the specific roles of these molecular determinants are still under debate [26, 27]. Therefore, the elucidation of the mode of action of these virulence factors toward the host cell is of utmost importance to accomplish a more comprehensive view of the host-parasite interactions as well as immune modulation and thus also focus a new insight of target molecules for therapeutic intervention of leishmaniasis.

2 Proteases as Virulence Factors in *Leishmania*

Among the numerous parasitic virulence factors, parasite-derived proteases receive supreme importance due to their vital roles in the parasite life cycle and pathogenesis. Parasites produce a wide array of proteases which are essential for degradation of the tissue barriers for migration of parasites to specific sites, cleavage of host proteins for their essential nutrients, activation of inflammation that assure their survival and proliferation to sustain the infection [28–33]. A variety of *Leishmania* derived proteases have been shown clinically important for diagnosis and vaccine development. Several studies have illustrated metalloproteases, cysteine proteases, aspartic and recently serine proteases to be essential for *Leishmania* infection (Table 1).

The *Leishmania* genomes encode a large number of proteases [34]. *L. major* is expected to contain at least 154 peptidases (including aspartic-, cysteine-, metallo-, serine- and threonine-peptidases as well as one protease of unknown catalytic type) that represent around 1.8 % of the genome (Fig. 1) [34]. Comparative genomic analysis with the different species of the genus *Leishmania* have shown that the numbers of proteinase genes remain fixed among the various species (http://merops.sanger.ac.uk/).

Table 1 Biological roles proteases in leishmaniasis

Classes of proteases	Biological name	*Leishmania* species	Immunological functions
Metallo-proteases	GP63	*L. donovani* *L. amazonensis* *L. mexicana* *L. major*	Inactivates IgG by hydrolysis, evade complement-mediated lysis by cleaving of C3b into C3bi, degrades NF-κB and inhibit IL-12 and NO expression, protects against antimicrobial peptides, affects the natural killer (NK) cell functions, alters signaling by SHP-1 activation leading to inhibition of JAK2/STAT1α pathway, IRK1 kinases and MAPK, degrades JNK, hydrolyses MARCKS/MRP, cleaves mTOR, inactivates transcription factors by cleaving c-Jun and AP-1 [7, 14, 15, 18, 21, 22, 25, 37–40].
	Intracellular metalloprotease (MP-Ld)	*L. donovani*	MP-Ld located extensively near the flagellar pocket region, appears to play important roles in parasitic development [65]
Cysteine proteinases	CPA	*L. infantum*	Plays a role in infection of mammalian host cells [77]
	CPB	*L. mexicana* *L. major* *L. amazonensis* *L. chagasi* *L. pfanoi*	Induces Th2-associated immune response, induces lesions; inhibits of IL-12 production by degrading NF-κB, inhibits of NO generation by cleaving STAT-1 and AP-1 transcription factors, degrades of IκB-α, IκB-β transcription factors, prevents antigen presentation by degrading MHC class-II molecules, inhibits NK cells proliferation and cleaves CD4 glycoprotein of human T cells [36, 62, 67–69, 88, 95]
	CPC	*L. mexicana* *L. chagasi*	Subverts microbicidal effects of macrophages and contributes to resistance, induces TGF-β expression in human cell culture [36, 75, 90, 91]
Serine proteinases	Oligopeptidase B	*L. donovani* *L. mexicana* *L. major*	Help to differentiate from promastigote to amastigote. Regulate levels of enolase on the parasite cell surface and facilitate parasite entry into macrophages. Contributes to retain macrophages infection [111, 121, 193]
	Subtilisin type seine protease	*L. donovani*	Plays essential role in promastigotes to amastigotes differentiation, detoxifies reactive oxygen intermediates and maintains redox homeostasis, and is essential for *Leishmania* virulence [136]
	Serine protease	*L. amazonensis*	Enhances the *Leishmania* infection by promoting Th2-type of immune responses and is essential for parasite survival [138, 190]
	115 kDa Serine protease (pSP)	*L. donovani*	Associated with metacyclic promastigotes, located mainly at flagellar pocket region, confers significant protection via IFN-γ induced down-regulation of TNF-α mediated MMP-9 activity in experimental visceral leishmaniasis [140]
	Intracellular serine protease (SP-Ld)	*L. donovani*	Localized in the flagellar pocket as well as at the surface of the parasite, it down regulates the phagocytic activity of macrophages [65]
Aspartic proteases	Presenilin 1 (PS1) type	*L. major*	Cleaves type I membrane proteins and effectively involved in autophagy [66]
	Signal peptide peptidase (SPP) type		Cleaves the transmembrane domains of signal peptidases [66]
	Ddi1-like protein	*L. major* *L. mexicana*	Essential for *Leishmania* growth and macrophages infection [142, 146, 147]

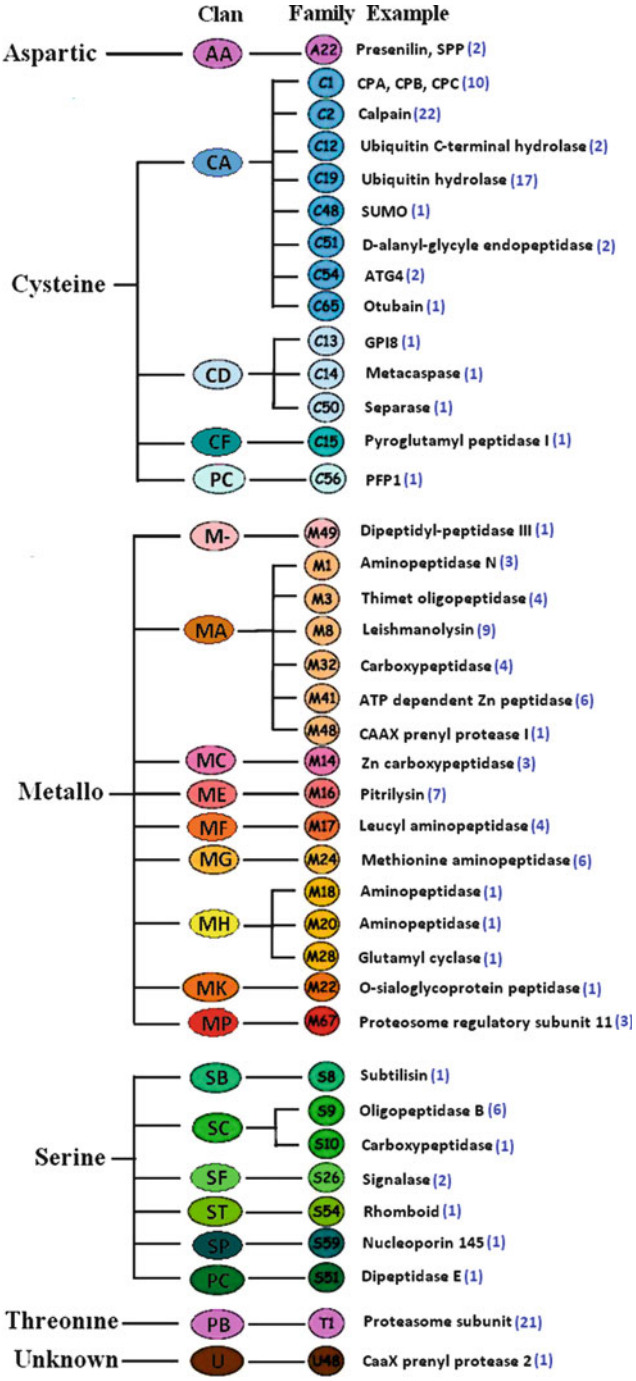

Fig. 1 Clans and families of *L. major* peptidases. Nomenclature is done on the basis of the MEROPS database (http://merops.sanger.ac.uk/). The estimated number of peptidases in each family is represented by *numbers* within *brackets* [34]

Many proteases are reported in both forms of *Leishmania* and their roles in the parasite physiology and immunoinvasion have been elucidated. A number of metalloproteases and cysteine proteases have been suggested to be virulence attributes that contribute to *Leishmania* pathogenesis by modulating the host cell signaling [25, 31–39]. Although, serine proteases have been extensively studied because of their imperative roles in parasite survival and pathogenicity [25, 39–44], roles of serine proteases in leishmaniasis have not been investigated adequately. In this context, the present review deals with the leishmanial proteases with special reference to the serine proteases, their possible involvement in *Leishmania* pathogenesis and to consider them as promising drug target for the prevention of the disease.

2.1 Metalloproteases

Promastigote major surface protease (MSP) leishmanolysin or GP63, the most abundant surface glycoprotein of *Leishmania*, belongs to the clan MA, M8 family of endopeptidases [34]. Its abundant expression of *Leishmania* promastigotes surface is well explored and its presence in amastigotes is also known [45]. It is bound to the surface membrane by a GPI (glycosyl phosphatidyl inositol) anchor which can be cleaved in vitro by phospholipase C (PLC) but its release *in vivo* depends on autoproteolysis [46]. *L. braziliensis* alone has at least ninety-seven metalloproteinase (http://tritrypdb.org, http://blast.ncbi.nlm. nih.gov/) and 16 families of metallopeptidases were identified in *L. major* (Fig. 1) [34]. GP63, accounting for about 1 % of the total protein in promastigotes of *Leishmania*, are potentially important during different stages of the life cycle [37]. GP63 share several characteristics with mammalian matrix metalloproteases (MMPs) that includes degradation of the extracellular matrix, cell surface localization, activation by Zn^{2+} and inhibition by several chelating agents and α-2-macroglobulin and like MMPs, it has also a wide range of substrates including casein, gelatin, albumin, haemoglobin, and fibrinogen [37, 47].

The zinc dependent metalloprotease GP63 can act in the sandfly midgut as well as macrophages parasitophorous vacuoles. In the amastigote, leishmanolysin (GP63) is located in the large lysosomes [48, 49]. At least 18 metalloproteinase genes were detected in *L. chagasi* and seven metalloproteinase genes have been identified in *L. major* [46, 50]. The up-regulation of GP63 expression in invasive metacyclic promastigotes suggests that the protease plays an important role in the early stages of infection of the mammalian host [51]. Examination of *Leishmania* strains expressing varying levels of GP63 has suggested that it participates in direct binding of the parasite to macrophages [22, 39]. Besides its presence in different species of *Leishmania*, GP63 has also been reported in various trypanosome species like *Crithidia fasciculata*, *T. brucei*, and *T. cruzi* [37]. More recently leishmanolysin homologs have been found in *Trichomonas vaginalis* as well [52].

GP63 plays important diverse roles in leishmanial pathogenesis. It helps *Leishmania* promastigotes to evade complement-mediated lysis (CML) by proteolytic cleavage of C3 complement into inactive iC3b (inactive C3b) thereby helping

the parasite to avoid complement pathway and thus enhances phagocytosis of promastigotes by host Mø through macrophage receptors CR3 [53]. It also favors promastigote migration through the extracellular matrix (ECM) by degrading the extracellular matrix components such as fibronectin and collagen IV and thereby further facilitates the parasite adherence to macrophages [54]. As an immunemodulator, GP63 diminishes both T cell responses either by cleaving CD4 molecules from T_h cells or also by degrading many other intracellular peptides presented by major histocompatibility complex class I (MHC-I) molecule [55, 56]. Additionally, GP63 mediated enzymatic degradation of antimicrobial peptides causes resistance of the parasite to apoptotic killing by these peptides [57].

GP63 modulates host negative regulatory mechanisms by degrading various kinases and transcription factors. It is responsible for the hydrolysis of the myristoylated alanine-rich C kinase substrate related protein (MRP), a major PKC substrate in macrophages and thereby inhibits PKC activation [58]. Recently it has been proposed that GP63 dependent alternative mechanism could be involved in PKC alteration [22]. It was also reported that *Leishmania* GP63 is able to rapidly reach the intracellular milieu of the host macrophage through lipid raft and activate host protein tyrosine phosphatases (PTPs) [18, 38]. It induces activation of the protein tyrosine phosphatase (PTP) SHP-1 through a lipid raft-based mechanism causing inhibition of JAK/STAT pathways, IFN-γ stimulation and reducing NO production [22, 59]. Thus the progression of leishmaniasis involving PTPs activation also requires the proteolytic mechanisms involving GP63.

Furthermore, GP63-mediated SHP-1 activation involves MAPK inactivation where JNK kinase and its downstream signaling target namely c-Jun, is cleaved by GP63 thus directly affecting MAPK activation [60]. At the same time, GP63 mediates cleavage of p65subunit of the NF-κB into a smaller subunit (p35) that enters the host cell nucleus and triggers the expression of chemokines [61]. Taken together, GP63 was found not only to degrade NF-κB completely but also implicated in the proteolysis of c-Jun, the central component of the transcriptional complex AP-1 leading to decreased IFN-γ-induced NO production [60, 62].

Natural killer (NK) cells play important roles in innate immunity via cytotoxic activity and early cytokine production against pathogens, including parasites. Proliferation, receptor expression and IFN-γ released by natural killer (NK) cells have been shown to be affected by *Leishmania* GP63 therefore inhibiting the Th1 type immune response with parasite infection and has also been shown to cleave mTOR to control translational system of host cells [63, 64]. Destabilizing the proper functioning of the transcriptional machinery by *Leishmania* GP63 results in the enlistment of the macrophages to serve as host, thereby precluding the expression of host factors such as IL-12 and iNOS that threaten their survival [22, 38, 39]

Interestingly, another metalloprotease (MP-Ld) was also identified in *L. donovani* promastigotes [65]. Both immunofluorescence and immune-gold electron microscopy studies revealed that MP-Ld is located extensively near the flagellar pocket region (Fig. 2). It seems to be of collagenase type as it degrades azocoll with maximum efficiency. It was predicted that MP-Ld played major and important role in parasitic development rather than in the infection process.

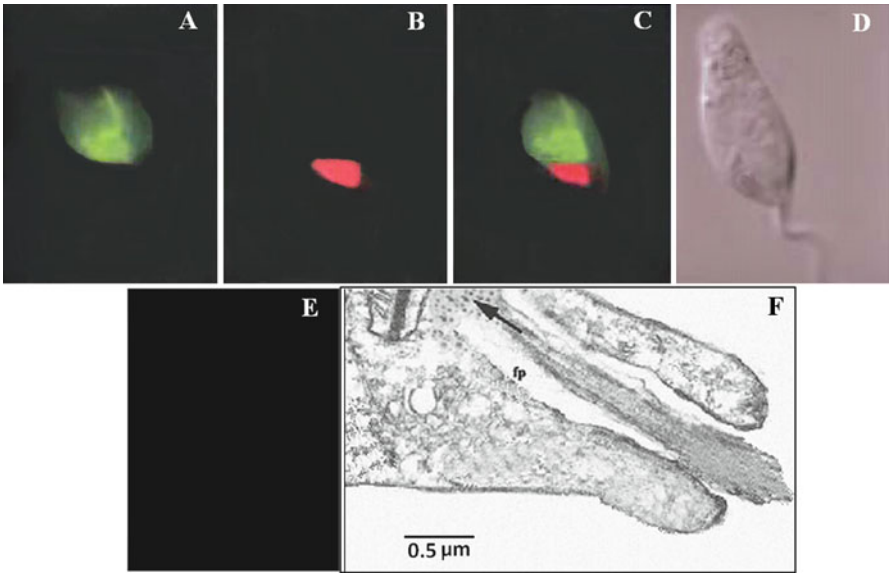

Fig. 2 Localization of *L. donovani* intracellular metallo protease (MP-Ld) by confocal immunofluorescence and immune-gold electron microscopy. MP-Ld was envisioned by FITC-labeling (**a**), TRITC conjugated con A was used to label the flagellar pocket (*red*) (**b**), Merged images showing co-localization of MP-Ld within the flagellar pocket (**c**) and (**d**) is the phase contrast image of MP-Ld. No fluorescence was detected in presence of corresponding pre-immune serum (**e**). *L. donovani* promastigotes showing the presence of gold particles (**f**) indicate distribution of MP-Ld near the flagellar pocket denoted by the *arrow* [65]

Overall view of these evidences suggest that metalloproteases of *Leishmania* are of significance that could unveil the molecular mechanisms of host-parasite interactions where GP63 is a profound virulent factor of *Leishmania* and could be an effective target of novel therapeutic and prophylactic approach for prevention of leishmaniasis.

2.2 Cysteine Proteases

Leishmania expresses many distinct genes (Fig. 1) encoding a total of 65 cysteine proteases and they are involved in a wide range of important biological processes [36]. *L. major* was found to have members of four clans of cysteine peptidases, consisting of enzymes of eight families of clan CA, three families of clan CD and one family each of clans CF and PC [66]. Cysteine proteases (CPs) have been demonstrated as important virulent factor as they are essential for *Leishmania* survival, replication, development, metabolism, host cell infection and evasion of host immune response [36, 67].

The proteases of the papain family (clan CA, family C1) are the most extensively studied proteases of *Leishmania*. The best characterized of these enzymes are the cathepsin L-like A (CPA) and B (CPB) families of cysteine proteases and the cathepsin B-like C (CPC) family of cysteine proteases, all of which are lysosomal in amastigote stages [66]. Many studies have identified CPs as prevalent virulence factors in *Leishmania* genus [36]. It has been investigated that both CPB and CPA facilitates effective autophagy and differentiation of the *Leishmania* [68]. The proteases have been demonstrated as potential drug targets and vaccine candidates [66, 69–71] as they are essential for the growth of *Leishmania* and for the progression of lesions.

Roles of these proteases in the modulation of host immune response have been reported. The lack of both CPA and CPB lead to increased production of Th1-type cytokines response, and reduced production of IL-4, a signature cytokine of the Th2-type immune response [72–74]. CPC has also been found to exacerbate the disease [75]. There is evidence that CPC can also play a relevant role as a key *Leishmania* virulence factor as it may contribute to some of the immunoregulatory activities of *L. chagasi* by inducing TGF-β expression [36, 75, 76]. Based on gene suppression studies CPA from *L. infantum* was found to be responsible for virulence of the parasite [77, 78].

Cathepsin L-like proteases of *L. pifanoi* and *L. mexicana* and the cathepsin B-like protease of *L. major* localized in lysosomes implicates the involvement of these enzymes in protein degradation [36, 70, 79]. Cathepsin-L like cysteine protease (CPB) promotes a Th2-type of immune response by cleaving the IL-2 receptor CD25 and the low-affinity IgE receptor CD23 [73, 79, 80]. Moreover, active participation of leishmanial CPs in T-cell mediated immunity is due to the presence of T-cell epitope at the COOH-terminal region of the protease itself [81, 82]. In addition, computational analysis of *Leishmania* CPs also reveals that they contain potential epitopic regions [83]. Due to immunogenicity of CPs, they have been used as vaccine candidates with different degree of protection in animal model [84–87]

A high CP activity was observed in extract of *L. amazonensis* amastigotes, but promastigotes from the exponential or stationary phases exhibited very low proteolytic activity [88–90]. In several species of *Leishmania*, there is an association between the level of CP expression and virulence [72, 91, 92]. CP is highly expressed in amastigotes and very low level in metacyclic promastigotes which might specify its central roles for intracellular survival of the parasite [36]. Correspondingly, *Leishmania* lacking cysteine proteases or parasites treated with specific cysteine protease inhibitors massively exhibits less infectivity [92–94].

Leishmania CPs have also been implicated in the inhibition of the crucial role of host cysteine proteases in the procession of antigen presentation by degrading MHC class II molecules in the parasitophorous vacuole [95] and partially inhibit host immune response. *Leishmania* amastigote CPs may also involve positive alteration of PKC mediated signaling that causes an enhanced expression of MKP3, PP2 and MKP1favoring intracellular survival [16, 22]. Another important function of CPs as virulent factors is the degradation of the transcription factors STAT1 and AP-1 which subsequently hinder NO production in host macrophages [96]. Alternatively, *Leishmania* CPs are also capable to disrupt NF-kB signaling by drastic cleavage of

NF-κB family proteins with downregulation of IL-12 production and concomitant persistence of infection in host macrophages [62, 97].

Collectively, *Leishmania* CPs are considered as a key factor with potential attribute in disease pathogenesis and hence might be addressed for developing a suitable drug of leishmaniasis.

2.3 Serine Proteases

Serine proteases are extensively dispensed in nature i.e. in all cellular organisms and more than one third of all known proteolytic enzymes are serine proteases grouped into 13 clans and 40 families [98]. They are a diverse group of enzymes that are characterized by the presence of three critical amino acids-histidine, aspartate, and serine-in the catalytic site [99]. These residues form together the "catalytic triad" of serine proteases. The family name is originated from the nucleophilic 'Ser' in the enzyme's active site, which attacks the carbonyl moiety of the substrate peptide bond to form an acyl-enzyme intermediate thus to hydrolyze peptide bonds [100]. The "catalytic triad" is associated with many families of seine proteases including the trypsin, subtilisin, prolyl oligo peptidase and serine carboxypeptidase families [101].

Serine proteases can be classified into three groups based mainly on their primary substrate preference: (1) trypsin-like, (2) chymotrypsin-like and (3) elastase-like. Trypsin family proteases represent the most abundant group in vertebrates, where they function in blood coagulation, the complement cascade, intestinal digestion, in inflammatory responses, reproduction and many other physiologic processes as in development, maintenance, and pathology of the nervous system [102–105].

In general, serine proteases of protozoan parasites and some bacteria are of the subtilisin (SB) type and in many cases oligopeptidase B (OPB) type. Chymotrypsin, trypsin and elastase (trypsin family) share closely-similar structures containing active serine residue at the same position (Ser-195), while subtilisins have Ser residue at 221. Subtilisin (serine endopeptidase) is a non-specific protease.

In the trypanosomatids, serine protease research has generally centered on the oligopeptidase B (OPB) and prolyl oligopeptidase (POP) [41, 106]. During entry into the host cell, it is supposed that *Trypanosoma cruzi* OPB augments host cell penetration by eliciting Ca^{2+}-signaling mechanism [42, 107]. *T. cruzi* prolyl oligopeptidases (POP) may be important to degrade extracellular matrix proteins such as collagen and fibronectin to facilitate parasite invasion process [108] as the penetration of *T. cruzi* into host is reduced in the presence of selective exogenous OPB and POP inhibitors [32, 109–111].

Serine proteases in apicomplexans have mainly centered on protein processing and other functions related to intracellular survival [112]. To date, many serine proteases have been found to be essential virulence factors in protozoan parasites including *Plasmodium falciparum*, *Eimeria tenella*, *Toxoplasma gondii*, *Babesia divergens*, *Perkinsus marinus* etc. [44, 112–118]. *Helminthes* and *Schistosomes* parasites exploit their serine proteases in anticoagulation and invasion respectively [32].

Excluding metallo and cysteine proteases, *Leishmania* also contain at least twenty-three serine proteinases (http://tritrypdb.org, http://blast.ncbi.nlm. nih.gov/). The activity of a serine peptidase was first purified and characterized from soluble extracts of *L. amazonensis* promastigotes [119]. This serine peptidase was characterized as an oligopeptidase as it can't hydrolyze proteins or large peptides, but it cleaves only small peptides substrates, at their carboxyl side. *Leishmania* OPB was subsequently described in *L. major* in 1999 [120]. By means of mass spectrometry and gene deletion approach, the *Leishmania* oligopeptidase B (OPB; Clan SC, family S9A), was identified and characterized [121]. The OPB activity was detected in both promastigote and amastigote stages of *Leishmania*. However, this activity was significantly elevated in the amastigote stage for both *L. donovani* and *L. Mexicana*. The *L. amazonensis* OPB was cloned and sequenced and was found to be 90 % identical to *L. major* and *L. infantum* OPB and 84 % identical to *L. braziliensis* [122]. It is important to keep in mind that *Trypanosoma* species do not express enzymes showing serine protease activities, but only serine oligopeptidases with specific functions in many steps of mammalian cell invasion [123, 124].

Furthermore, some serine proteases have been reported in *Leishmania* in secreted as well as in intracellular form [65, 125–131]. A detergent soluble 110 kDa serine protease [125] and a 68 kDa intracellular serine protease in aqueous extract were identified and characterized from *L. amazonensis* promastigotes [126, 132]. At the same time extracellular serine proteases from different species of *Leishmania* like *L. amazonensis* [127], *L. braziliensis* [128] and more currently from *L. donovani* [130] have been demonstrated with similar biological properties and location near the flagellar pocket region in promastigotes and megasomes of amastigotes [128, 133, 134].

L. chagasi, the causative agent of visceral leishmaniasis in Latin America also shows serine protease activities [135]. Three serine proteases named as LCSI, LCSII and LCSIII were isolated from extract of *L. chagasi* [129] exhibiting similar compartmentalization and substrate specificities with the serine proteases of other *Leishmania* species.

The role of serine proteases in visceral leishmaniasis caused by *L. donovani* is little known. An aprotinin sensitive *L. donovani* extracellular serine protease (pSP) of molecular mass 115 kDa was first identified [130] with their location in the flagellar pocket region in promastigotes and amastigotes (Fig. 3) [134]. Flow cytometry (Fig. 4) and confocal immunofluorescence (Fig. 3) analysis also revealed that the expression of the protease diminishes sequentially from virulent to attenuated strains of this species and is also highly associated with the metacyclic stage of *L. donovani* promastigotes [134]. Importantly pSP is upregulated during metacylogenesis and hopefully makes them important candidate as a participant in host-parasite interaction. Moreover, the pSP has strong proteolytic activity against extracellular matrix proteins, such as collagen and fibronectin [130], which suggests that the protease might be a superior agent for host tissue invasion and thus the role of pSP in host infection appears to be significant.

Besides the expression of secreted serine protease, a novel intracellular serine protease (SP-Ld) was also identified in *L. donovani* promastigotes [65]. This intracellular SP-Ld is also concentrated in the flagellar pocket region as well as on the surface

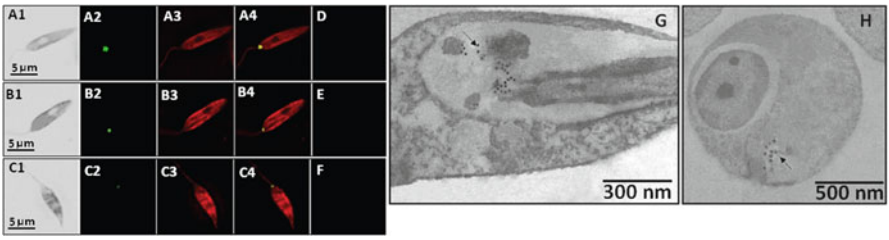

Fig. 3 The immunofluorescence images of Early-passage (*A1–A4*), late-passage (*B1–B4*), and UR6 (*C1–C4*) promastigotes of *L. donovani*. The promastigotes labeled for the pSP are shown in the *green* channel (*A2*, *B2*, and *C3*): GP63 are shown in the *red* (*A3*, *B3* and *C3*), Merged image in *yellow* channel (*A4*, *B4*, and *C4*). The phase-contrast image is shown on the *left* (*A1*, *B1* and *C1*). No Fluorescence was detected in similar preparations reacted with the preimmune serum (**d**, **e**, and **f**). Intracellular localization of the pSP of *L. donovani* by immunogold electron microscopy; the presence of gold particles in the flagellar pocket regions of the parasites Promastigotes (**g**) and amastigotes (**h**) indicated by *arrows* [134]

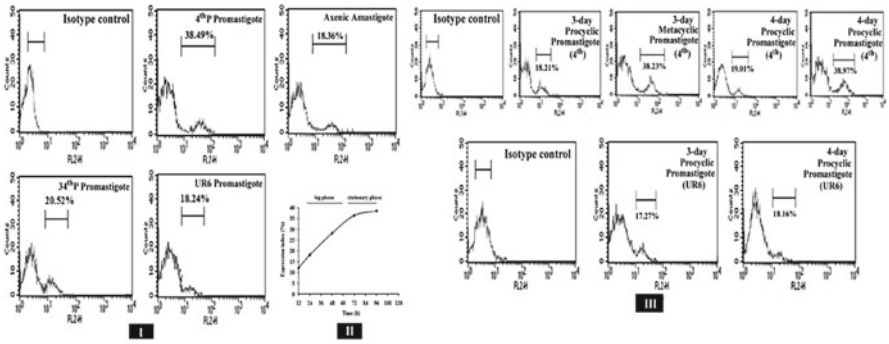

Fig. 4 Flow cytometric analysis of expression of the pSP. (*I*) Fluorescence histograms show the expression levels of the pSP in 4th-P, 34th-P, and UR6 promastigotes and axenic amastigotes of *L. donovani*. (*II*) Expression of the pSP of 4th-P promastigotes of *L. donovani* at different phases of growth. (*III*) Fluorescence histograms showing the expression levels of the pSP at procyclic and metacyclic stages of virulent promastigotes and at procyclic stages of attenuated UR6 promastigotes at 72 and 96 h of culture [134]

of the parasite (Fig. 5). The major role of SP-Ld could be predicted in invasion process as it down regulates the phagocytic activity of macrophages (Fig. 6) [65].

Using biochemical and molecular strategies two other serine proteases were also identified and characterized in *L. donovani* promastigotes which are of subtilisin [136] and oligopeptidase B [121] type.

During differentiation from promastigote to amastigote, OPB is upregulated in *Leishmania* and regulate levels of enolase on the parasite cell surface facilitating parasite entry into macrophages [121]. The direct effect of OPB on the host immune system was shown by examining the effect of infection with an OPB mutant strain on the expression of host genes. Infection of macrophages with a wild type

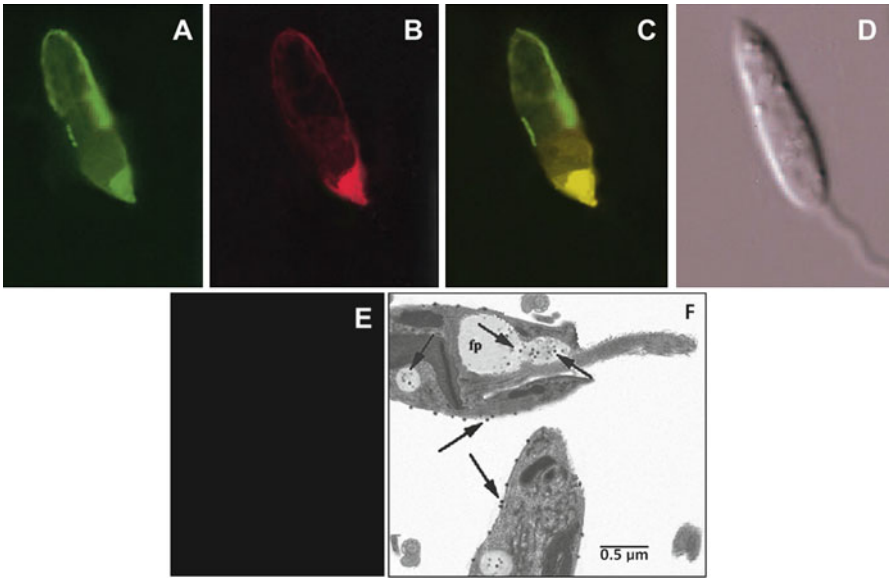

Fig. 5 Localization of *L. donovani* intracellular serine protease (SP-Ld) by confocal immunofluorescence and immune-gold electron microscopy. SP-Ld was envisioned by FITC-labeling (**a**), TRITC conjugated con A was used to label the flagellar pocket (*red*) (**b**), Merged images showing co-localization of SP-Ld within the flagellar pocket (**c**) and (**d**) is the phase contrast image of SP-Ld. No fluorescence was detected in presence of corresponding pre-immune serum (**e**). The presence of gold particles represents SP-Ld within flagellar pocket, cytoplasmic vesicles as well as at the surface of the *L. donovani* promastigotes indicated by the *arrows* (F) [65]

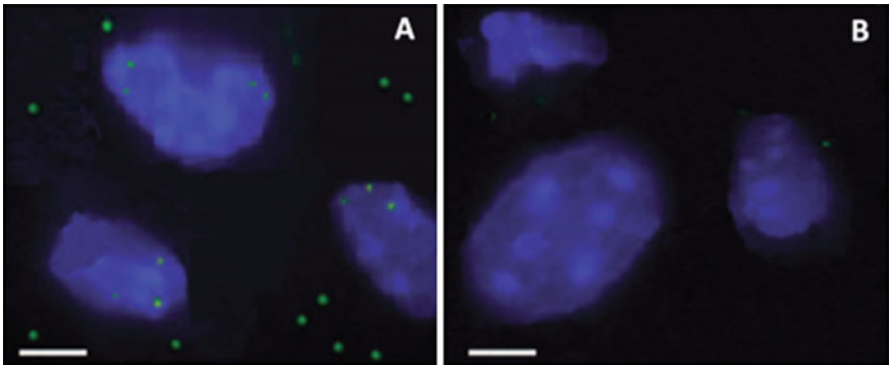

Fig. 6 Phagocytic activity of macrophages. FITC-coupled latex beads were co-incubated with macrophages in absence (**a**) and in presence of anti SP-Ld (**b**) treated parasites. Scale bars, 1 **µ**m [65]

L. donovani strain alters expressions of 23 genes, but infection with a mutant strain in which the oligopeptidase B gene was deleted leads to changes in 495 genes. This proves that OPB is necessary for *Leishmania* to silently infect macrophages [121]. Furthermore, these OPB (−/−) parasites displayed decreased virulence toward

mammalian host and suggested that *Leishmania* OPB itself is a prevalent virulence factor and also acts in conjunction with other factors [137].

The subtilisin protease (SUB; Clan SB, family S8) from *Leishmania donovani* was found to possess a unique catalytic triad and SUB-deficient *Leishmania* displayed reduced ability to undergo differentiation from promastigote to amastigote with some deformities i.e. abnormal membrane structures, retained flagella and increased binucleation [136]. On the basis of proteomic analysis, it has been reported that subtilisin is the maturase for tryparedoxin peroxidases to detoxify reactive oxygen intermediates for the maintenance of redox homeostasis and that is essential for *Leishmania* virulence [136]. Moreover, the activity of this serine protease is higher by several folds in amastigotes compared to promastigotes, suggesting an important role for this enzyme in parasites inside the host cells [136].

Serine proteases from *L. amazonensis* directly activated Th2 type immune response, and increased susceptibility to infection but, this effect was successfully eliminated in presence of specific serine protease inhibitors but not cysteine protease inhibitors [138]. So, *L. amazonensis* serine proteases exaggerate the infection by promoting Th2 type immune response. It was predicted that the *L. amazonensis* amastigote extract (LaE) containing serine protease is responsible for exacerbation of *Leishmania* infection by promoting Th2-type immune responses [139].

Besides being important targets of drug development against *Leishmania*, serine proteases are also vaccine candidates for leishmaniasis. Recently, Choudhury et al. [140] have shown that the *L. donovani* extracellular serine protease (pSP) confer significant protection in experimental visceral leishmaniasis (VL) and in this study the vaccine efficacy of pSP was further investigated for its prophylactic potentiality by regulating host MMP-9 profile. Hence, it can be postulated that *Leishmania* proteases may participate in modification of macrophages functions by modulating matrix metalloproteinase activity. Currently, available data suggest that serine proteases and MMPs might play essential functions in development of leishmaniasis and help researchers to investigate the miscellaneous roles of these proteases to design effective therapeutic strategies against leishmaniasis.

2.4 Aspartic Proteases

The presence of aspartic protease was first reported in *Leishmania* in 2005 [129]. It was present at its highest level in promastigotes and in the early stages of differentiation to amastigotes [135]. *L. major* genome contains two aspartic peptidases [66]. One has similar sequence with presenilin 1 (PS1), a multi-pass membrane peptidase, and is able to cleave type I membrane proteins [34]. Another one has identical sequence with an intramembrane signal peptide peptidase (SPP) which cleaves the transmembrane domains of signal peptidases [66]. PS1 has been implicated to be involved in autophagy in *L. major* [66]. An aspartic protease activity was also identified and characterized in *L. mexicana* promastigotes [141]. A recent study has reported that Ddi1-like protein is functional aspartyl proteinase in *L. major* and it

can be a possible potential target for novel antiparasitic drugs [142]. The antiproliferative effect of its inhibition makes this enzyme a putative new target for the development of leishmanicidal drugs. In this context, Savoia and co-workers in 2005 [143] first demonstrated the impressive effects of indinavir and saquinavir on the growth of *L. major* and *L. infantum*. Later, it was demonstrated that HIV aspartyl-protease inhibitors (HIV-PIs) powerfully reduce *L. infantum* infection in macrophages, either co-infected or not with HIV [144]. In addition, this activity was target of antiproliferative effect on *Leishmania* promastigotes and axenic amastigotes by HIV-PIs, Ac-Leu-Val-Phenylalaninal, saquinavir mesylate and nelfinavir [145]. A direct action of these HIV-PIs on *Leishmania* parasites opens an interesting standpoint for new drugs research development based on this novel parasite protease for the treatment of HIV/Leishmania co-infection [146]. In addition, HIV-PIs also hampered *L. amazonensis* growth and their interaction with macrophages, indicating that the HIV-PIs are active against a wide range of *Leishmania* species and probably induce several serious ultrastructural modifications in *L. amazonensis* promastigotes. This effect of HIV-PIs is terminated with parasite death, may be due to a disproportion between apoptosis and autophagy [147]. This dose-dependent inhibition of *Leishmania* aspartyl-protease activity by these drugs certainly validates the possible association between aspartic protease expression and basic molecular processes in *Leishmania*. Despite all these beneficial effects, the HIV-PIs induced an increase in the expression of CPB and GP63 [148]. So, further investigations are essential to control HIV/*Leishmania* co-infection. However, the noticeably increasing numbers of *Leishmania* and HIV co-infected patients and direct effect of the HIV-PIs on opportunistic pathogens support researchers to seek for direct effects of HIV-PIs on *Leishmania* [40, 149, 150].

3 Proteases as Drug Targets in Leishmaniasis

Leishmaniasis remains a challenge for public health due to lack of effective vaccine and thus as of now, chemotherapy is the only alternative for controlling the disease [26, 27]. The current treatments available are greatly disappointing due to high cost, toxicity and widespread resistance and therefore the present situation needs worldwide development of potential new drugs to combat leishmaniasis. One of the main features in the drug development is to identify a possible target of biological pathway of parasite life cycle, in a view of that the target should be either absent in the host or be unique from the host homologous proteins so that it can be exploited as a putative drug target. It has also been described that one of the characteristic features in the process of drug development is to identify the putative target [151]. In this context, proteases would be excellent objects because of their vital roles in parasitic biology [40, 152]. Hence, investigations are currently underway to elucidate their possible function by means of protease inhibitors. The protease inhibitors inactivate or block proteolytic enzymes by binding to its active site or by other mechanisms

[153] hindering one or several fundamental events caused by the enzymes and thus, uses of protease inhibitors also enhance our knowledge about the biological function of the enzymes in the parasite physiology. Therefore, the main approach has been to achieve good inhibitors of the target protease, in the faith that inhibition of the protease activity of the pathogen will be of therapeutic value.

Parenthetically proteases have being authenticated as druggable targets in many cases [27, 154–156], and protease inhibitors are also being broadly investigated to develop therapeutic drugs against cancer, cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases due to important roles played by the proteases in these diseases [157–160]. Hence, the ongoing progress in the design of protease inhibitors may also present a challenge for advanced therapies of parasitic diseases [155, 161–164]. Protease inhibitors thus have potential utility for therapeutic interposition in a variety of disease states including trypanosomasis and leishmaniasis [159]. However, as the parasites are eukaryotes, treatments of trypanosomatid diseases are difficult by means of protease inhibitors as antiparasitics because they may lead to the host toxicity and possible adverse side effects. But, current research on drug design makes it feasible for formulation of specific protease inhibitors with negligible cross reactivity and of great potentiality [40, 159].

For instance, the proteasome of *Leishmania* is a potential therapeutic target as inhibition of proteasome blocks parasite growth [165]. Besides, several research groups have suggested that cysteine proteases in *Leishmania* may also be very promising target [36, 71, 166, 167]. *Leishmania* treated with CP inhibitors showed reduced viability, growth and pathogenicity [70, 71]. In addition, treatment with a natural CP inhibitor, cystatin, promoted a protective response against *Leishmania* infection and a switch from a predominately Th2 to a Th1-type of immune response [72, 92, 93]. The CP inhibitors have also been shown to prevent the activation of a latent form of TGF-β, a known suppressive cytokine in *Leishmania* infections [168]. Moreover, it has been found that both metallo and cysteine peptidase inhibitors could hinder the growth of *L. braziliensis* as well as the association index with macrophages [169]. Thus, presently, a lot of researches are progressing to develop potent cysteine protease inhibitor as an antileishmanial drug. Unlike cysteine protease, a little research has been focused on *Leishmania* GP63 to evaluate it as a drug target. Previously, it has been demonstrated that development of higher affinity metalloproteases inhibitors may provide a novel approach for treatment of parasitic diseases [170]. *Leishmanias* lacking GP63 are unable to activate the host PTPs for sabotage host cell signaling and lose their ability to sustain infection [59]. Therefore, it can be clearly speculated that potent and specific inhibitors of GP63 could be able to trigger the host antimicrobial functions and thus would gain the accessibility of future antileishmanial therapeutics [40]. Nevertheless, due to immunogenicity and antigenicity of *Leishmania* GP63, many studies have been performed to evaluate its efficacy as vaccine against *Leishmania* [84, 171].

Currently, the possibility of implication of HIV protease inhibitors against *Leishmania* has raised interest due to increasing rate of HIV and *Leishmania* co-infection in certain regions of the world and protease inhibitors are being extensively used to treat HIV and *Leishmania* co-infected patients [172–175]. Recently,

some compounds are now being experimentally used to treat leishmaniasis targeting particular protease [176, 177].

Serine protease inhibitors have also been used by many investigators in parasitic diseases in searching potent drug [117, 178, 179] as serine protease activity can be regulated in the cells or in the organisms by employing specific protease inhibitors [180, 181]. These inhibitors are valuable tools for investigation of the biochemical properties and the biological functions of the proteases [182, 183]. In addition, invasion blockage of many parasites, including *Plasmodium falciparum* [184, 185], *Babesia divergens* [117], *Toxoplasma gondii* [186] and *Perkinsus marinus* [187] have been observed by using specific serine protease inhibitors. Previous reports have shown that pentamidine and suramin, exhibit trypanocidal activity through the inhibition of the cytosolic serine protease oligopepetidase B, a putative virulence factor in trypanosome [188, 189].

Initially, serine protease inhibitors were used to evaluate the possible functions of serine proteases in *Leishmania* [190]. The effect of serine peptidase inhibitors on the survival of *Leishmania* has shown that TPCK (N-tosyl-l-lysyl-chloromethylketone) and benzamidine both reduces viability and induce morphological changes in the *Leishmania amazonensis* promastigotes, raising the possibility that serine peptidases could be useful potential drug targets [190]. Moreover, treatment with different type of serine protease inhibitors, especially with aprotinin, which block the active site of the protease, resulted in marked reduction in cellular viability [190]. Specific doses of these compounds stimulate significant morphological modifications in the flagellar pocket region accompanied by forming bleb that coats the flagellar pocket [190]. These effects indicate that serine protease inhibitors are probably introduced through this structure and inhibited the serine proteases in this pocket region. Moreover, these serine proteases inhibitors induce the formation of autophagic vacuoles and destroy *Leishmania* promastigotes. Although serine proteases are essential for parasite survival, their function in *Leishmania* physiology remains to be illuminated. However, this is the first evidence where *Leishmania* serine proteases have been emerged to be another promising target for the development of antileishmanial chemotherapy. Moreover, the treatment of *L. amazonensis* promastigotes antigens (LaAg) with irreversible serine protease inhibitors reversed its disease-promoting effect [138] which is again another indication of exploitation of serine proteases in the development of antileishmanial drugs [152, 190, 191].

Although, several *Leishmania* serine proteases have been characterized, their function in the parasite physiology is still under investigation. Based on studies from our laboratory and other groups, serine proteases seem to play essential roles in infection process and deactivating the macrophage during the initial interaction between the host and the parasite [65, 121, 134, 136–140]. Altogether, these characteristics of *Leishmania* serine proteases strongly suggest that potent and specific inhibitors of serine protease could instigate the activation of host's antimicrobial properties and thereby leading to destruction of parasites. Hence, the possible approach of using specific serine protease inhibitors as prophylactic drugs could be able to inhibit the onset of *Leishmania* infection [40].

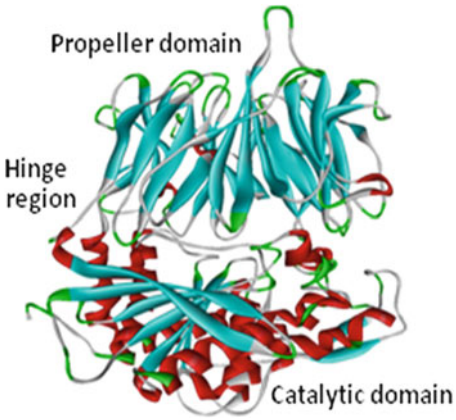

Fig. 7 Crystal structure of OPB [PDB ID 2XE4] done by McLuskey et al. [111]: The tertiary structure of OPB with antipain bound in the active site. General loop regions are shown in *yellow*. The hinge regions are between the two domains and the catalytic domain is represented by deep colors

But, uses of serine protease inhibitors in treatment of leishmaniasis need extensive studies to understand the roles of serine proteases in parasite physiology and pathogenesis. In some reports, it has been postulated that structure based drug design could be achieved by means of three dimensional model e.g. in *L. amazonensis* oligopeptidase B (Fig. 7) [111]. It has also been proposed that prolyl oligopeptidase and oligopeptidase B, both members of the S9 serine protease family would be admirable choice for drug design against Chagas disease, leishmaniasis and African trypanosomiasis [192, 193]. In addition to the computational design, development and optimization of a suitable protease inhibitor, based on the 3D structure of the target protease will be valuable tools for investigation of the biochemical properties and functions of proteases as well as in the treatment of leishmaniasis. On the other hand, ongoing researches on *Leishmania* proteases are still expanding our knowledge on parasite biology, particularly with a great concern over current concept of serine proteases and demonstrating them to be potential drug targets. Therefore, as a whole, the developments of both synthetic and natural protease inhibitors have relevant importance in the search of new therapeutic alternatives for leishmaniasis. Eventually, the development of protease inhibitors of particular parasitic proteases will be the best option for improved understanding of physiological significance of the proteases in disease pathogenesis as well as to identify them as good candidates for antileishmanial therapy.

4 Conclusion

Leishmania gets advantages from various virulent factors especially from proteases. leishmanial proteases help invasion and survival in intra or extracellular environments of the host. So, proteases are considered as potential drug targets in *Leishmania*

parasite For instances, *Leishmania* GP63 and cysteine protease subvert host immune response throughout various mechanisms. Aspartic protease is an important virulent factor in case of *Leishmania*-HIV co-infected patients. In this context, roles of *Leishmania* serine proteases need to be further defined because some recent reports suggest that this protease also perform crucial roles in parasite physiology and in the host-parasite interaction. For instance, Oligopeptidase B (OPB) is increasingly being implicated as an important virulence factor in leishmaniasis. Elucidation of the substrate specificity and regulation of OPB activity paved the way to develop drugs that are specific for the parasite. It was also observed that specific serine protease inhibitors alters parasite morphology and reduced the viability, growth of *Leishmania* and also causes death of both extracellular and intracellular parasite. Hence, protease inhibitors must be considered to be promising candidates for drug development in the leishmaniasis treatment and it would be a rational approach toward other parasitic diseases as well.

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An Updated Inventory on Properties, Pathophysiology and Therapeutic Potential of Snake Venom Thrombin-Like Proteases

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Abstract Venom from viperidae family of snakes contains highly specific proteases affecting the blood coagulation mechanism and hence interferes with the haemostatic mechanism of victim. A special class of venom serine proteases functionally resembles with thrombin (coagulant serine proteases) is known as Snake Venom Thrombin-like Enzymes (SVTLEs). The coagulant serine proteases are able to clot fibrinogen in in vitro condition by virtue of their ability to release either fibrinopeptide A, or B or both A and B from fibrinogen however, in in vivo condition they cause defibrinogenation of plasma. Despite a high degree of similarity in primary structure, a minor variation or substitution in the subsites of the active site of the SVTLEs may lead to differences in the substrate specificity amongst these enzymes resulting in displaying different biological activity by these enzymes. The SVTLEs are mostly glycoprotein in nature however; contradictory and fragmentary data are available concerning the role of glycosylation on biological activity of these enzymes. During the last few decades, SVTLEs are subject of intensive research for their possible therapeutic application in the treatment of hyperfibrinogenemia related disorders as well as development of diagnostic reagents for clinical study. The present review provides up to date information available on structure-function properties, pathophysiology and therapeutic application of SVTLEs covering a period from 2007 to till date.

Keywords Thrombin-like enzyme • Coagulant enzyme, serine protease • Snake venom • Defibrinogenation • Fibrinopeptide release • Thrombosis

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1 Introduction

Snake venom contains many biologically active proteins, polypeptides and some non-enzymatic toxins which may interfere with the haemostatic mechanism of victim, the primary object of which is either to immobilize and/or kill the prey before being swallowed or as a defense mechanism of snake. Proteases are amongst the well characterized enzymatic components of the snake venom responsible for exerting a variety of toxic effects mostly by inducing the disorder of the host coagulation system in addition to exerting tissue-hemorrhage, myotoxicity, activation of complement system, platelet aggregation and edema inducing activity [1–4]. In a broad sense, venom proteases may be classified as serine proteases, cysteine proteases, aspartic proteases and metalloproteases [5]. The serine proteases belong to the trypsin family S1 of clan SA and represent the largest family of peptidases [1]. A special group of snake venom serine proteases, functionally and may be structurally similar to thrombin, show in vitro fibrinogen clotting activities are known as snake venom thrombin-like enzymes or SVTLEs [6–8]. Nevertheless, SVTLEs acting as defibrinogenating agents in vivo reduce the fibrinogen content of plasma of the treated or envenomed animals [9]. Since hyperfibrinogenemia is considered as one of the major risk factors for the onset of cardiovascular ailment such as intravascular thrombosis and it also enhances the risk of onset and progression of vascular diseases [10]; therefore, SVTLEs showing potent defibrinogenating activity have been considered as candidates for the treatment of hyperfibrinogenemia associated disorders [7–9, 11]. The most appropriate examples of commercially explored SVTLEs include Defibrase from *Bothrops atrox*, Viprinex from *Agkistrodon rhodostoma* and reptilase from *Bothrops jararaca* venom which are under clinical trials [11].

A survey of literature shows that the study on the coagulation effect of ophidian venoms was started as early as in 1957 and since then many reports on SVTLEs are published [7, 8, 11]. However, during the last few decades, many of such enzymes are subject of intense research interest from the standpoint of their structure-function relationships as well as their therapeutic and diagnostic applications. Many of the excellent review articles have well described the structure-function properties, catalytic mechanism and biological activities of SVTLEs isolated mostly before 2006 [6–8, 11]. After 2006, more than 20 SVTLEs have been purified and characterized. Considering the urgent need to fill the gap of knowledge on recent advances on SVTLEs research, the present article will focus on this important class of biomolecule isolated and characterized from snake venom from 2007 to till date.

2 A Brief Account on the Catalytic Mechanism of SVTLEs

Like the other members of the serine protease family, the active site of the SVTLEs is made up of a catalytic triad of serine (Ser195), histidine (His57), and aspartate (Asp102) and this numbering was done according to the chymotrypsinogen system [12]. It has been proposed that a non-optimal Nd1-H tautomeric conformation

Table 1 A comparison of N-terminal sequences of SVTLEs reported in the literature from 2007

Name	Source	N-terminal sequence	References
Russelobin	*Daboia russelii russelii*	VVGGDECNINEHRSL	[14]
TLBan	*Bothrops andianus*	VI GGDECNINEHPFLVALY	[24]
Albolabrase	*Cryptelytrops albolabris*	VVGGDECNINE	[41]
Gyroxin	*Crotalus durissus terrificus*	VI GGDECNINEHRFLVALYE	[42]
Purpurase	*Cryptelytrops purpureomaculatus*	VVGGDECNINDHRSLVRIF	[25]
Bhalternin	*Bothrops alternatus*	VI GGDECNINEHRSLVVLF	[33]
TLBm	*Bothrops marajoensis*	VI GGDECNINEHRSLVVLF	[26]
BpSP-I	*Bothrops pauloensis*	VI GGDECDINEHPFL	[38]
Agacutin	*Agkistrodon acutus*	DSSGWSSYEGHEYYV (small subunit) DCSSGWSSYEEHQYY (large subunit)	[15]
BjussuSP-I	*B. jararacussu*	VLGGDE**C**DINEHPF**L**AFLYS	[17, 27]
TLE	*Bothrops asper*	VI GGDECNINEHRSLVVLFXSSGFL	[45]
DIII-4 BS3	*Bothrops insularis*	VVGGDECNINEHPFLVALYDG	[34]
BJ-48	*Bothrops jararacussu*	VVGGDXIPQVPFLAFLYSEYFX	[16]

possessed by His57 molecule is essential to exert the catalytic activity [12, 13]. An extensive hydrogen bonding network formed between the Nd1-H of His57 and Od1 of Asp102, as well as between the OH of Ser195 and the Ne2-H of His57 supports the catalytic triad for efficient catalysis [12]. The hydrogen bonds between Od2 of Asp102 and the main chain NHs of Ala56 and His57 are structural determinants to ensure the proper orientation of Asp102 and His57. In a nutshell, serine acts as a nucleophile and initiates the attack on the carboxyl side of the peptide, histidine acts as both a proton donor and an acceptor forming a tetrahedral imidazolium ion and acyl-enzyme intermediates, respectively. In the next step, histidine mediated deprotonated water molecule reacts with the acyl-enzyme complex followed by subsequent release of carboxylic acid product. The second step of deacylation is the rate limiting step of catalysis by snake venom serine proteases [12, 13].

3 Biochemical Properties of SVTLEs

From the year 2007 to till date, more than 20 of SVTLEs are purified by applying a variety of chromatographic techniques from venom of the many genera of snakes such as *Agkistrodon, Bothrops, Cryptelytrops, Crotalus* and *Daboia*. Table 1 shows the N-terminal sequences of SVTLEs whereas a summary of the biochemical properties of SVTLEs is shown in Table 2. For most of the SVTLEs, the first step is isolation of protease enzyme by fractionation of crude venom through size exclusion chromatography followed by purification of active enzyme either through reverse-phase high performance liquid chromatography (RP-HPLC) or by

Table 2 Some biochemical properties and substrate specificity of SVTLEs reported in the literature from 2007

Name	Source	Mass		pI	Optimum temperature and pH	% of total Venom protein	Glycogen content (%)	Substrate specificity	References
		SDS-PAGE	MS						
Russelobin	*Daboia russelii russelii*	51.3	38.7	nr	45 °C,9.0	0.5	56.2 (N-linked) 6.4 (neutral sugar)	N-Bz-Phe-Val-Arg-pNA, Nα-Tosyl-Gly-Pro-Arg-PNA, BAEE Fibrinogen	[14]
TLBan	*Bothrops andianus*	29 kDa (R) 25 (NR)	25,835.65 Da	nr	45 °C, 8.0	nr	~14 (sialic acid) ~24 (N-linked)	BAρNA FIBRINOGEN	[24]
Albolabrase	*Cryptelytrops albolabris*	33.7 kDa	nr	nr	nr	1.0	nr	N-benzoyl-L-PHENYLALANINE-L-VALYL-L-arginine p-nitroanilide, fibrinogen, BAEE	[1]
Gyroxin	*Crotalus durissus terrificus*	30 kDa	nr	5.5	nr	nr	nr	S-2238, S-2288	[42]
Purpurase	*Cryptelytrops purpureomaculatus*	35 kDa	nr	nr	nr	nr	nr	N-p-tosyl-glycine-l-proline-L-LYSINE *p*-nitroanilide BApNA Fibrinogen	[25]
Bhalternin	*Bothrops alternatus*	31.5 (R) 27 (NR)	nr	nr	nr	1.9	nr	Fibrinogen albumin	[33]
TLBm	*Bothrops marajoensis*	nr	33332.5 Da	nr	38–40 °C, 8.0	nr	nr	DL-BAρNA Fibrinogen	[26]
BpSP-I	*Bothrops pauloensis*	34 kDa (R) 30 kDa (NR)	NR	6.4	nr	3.1	~12 (sialic acid) ~26 (N-linked)	S-2288 S-2238 TAME	[38]
Agacutin	*Agkistrodon acutus*	30.9 kDa	29,402 Da	5.39	35 °C, 7.5	0.9		BAEE fibrinogen	[15]

Fibrinogen-activating enzyme	*Crotalus durissus terrificus*	30 kDa	nr	nr	nr	nr	nr	S2238	[19]
Do-	*C. d. collilineatus*	30 kDa	nr	nr	nr	nr	nr	S2238	[19]
BjussuSP-I	*B. jararacussu*	61 kDa	NR	3.8	nr	nr	~11 (sialic acid) ~39 (N-linked)	S-2238 and S-2288 TAME BApNA Fibrinogen Fibrin	[17, 27]
Coagulant serine protease (AH143)	*Agkistrodon halys*	30 kDa	nr	nr	37 °C,7.5	0.4	nr	Casein (–) BApNA (+)	[28]
TLE	*Bothrops asper*	32 kDa (NR) 24 kDa (R)	27,067 (± 27 Da).	nr	nr	nr	nr	Fibrinogen	[45]
Leucurobin	*Bothrops leucurus*	35 kDa	nr	nr	nr	3.5	nr	D-Phe-Pro-Arg-pNA	[35]
TLE	*Crotalus durissus terrificus*	33 kDa	nr	nr	nr	2.2	nr	nr	[44]
TLE (DIII-4 BS3)	*Bothrops insularis*	30 kDa	nr	nr	nr	nr	nr	nr	[34]
BJ-48	*Bothrops jararacussu*	52 kDa	48,036 Da	nr	45–50 °C, 7.5	nr	42 (n-linked) ~12 (Sialic acid)	TAME BApNA BAME Na-Tos–Gly–Pro–Lys-pNA	[16]

nr: not reported; R: reduced; NR: non-reduced

ion-exchange chromatography. For the isolation procedure, a number of substrates but mostly TAME, BApNA and S-2238 were used to assay the elution of active TLE from each chromatographic step. Nevertheless, the use of some other chromogenic substrates, for example, Bz-Phe-Val-Arg-pNA for determination of enzymatic activity of SVTLEs has also been reported [14]. Due to this different degree of substrate preference, till date no standard assay procedure could be described for SVTLEs. However, identity of most of the SVTLEs reviewed in this paper is confirmed by the N-terminal sequencing of protein and/or peptide mass fingerprinting of the tryptic digested peptide followed by MASCOT search of peptide masses in NCBI database.

Most of the SVTLEs reported from 2007 to till date are monomeric proteins; the only exception is Agacutin composed of two subunits of 15 and 16 kDa in its structure [15]. The molecular masse of most of the SVTLEs is observed within the range of 29–35 kDa and they represent 0.5–3.5 % of total venom proteins (Table 2). However, the molecular mass of some of the SVTLEs is found to be higher than 50 kDa, such as Russelobin (51.3 kDa), BJ-48 (52 kDa) and BjussuSP-I (61 kDa) (Table 2). The pI values of SVTLEs are determined within the acidic range. Snake venom is reported to contain both acidic and basic proteases; the function of the former group of proteases is to hydrolyze the proteins of the haemostatic system [1].

The N-terminal amino acid sequences of SVTLEs share significant homology and variations are observed only at a few positions (Table 1). For example, at position 2 V is replaced either by I or L. Similarly, the position 13 of SVTLEs is occupied either by R or P. However, the two remarkable variations in N-terminal sequence were observed for Agacutin from *Agkistrodon acutus* venom [15] and BJ-48 from *Bothrops jararacussu* venom [16]. The earlier studies have also demonstrated a significant similarity in the primary structure among the SVTLEs and a comparison of the primary structure of SVTLEs with those of classical serine proteases such as trypsin, chymotrypsin, thrombin and plasma kallikrein showed conserved regions among these enzymes [7, 8]. It has been documented that SVTLEs are more similar to kallikrein (34–40 %) followed by highly nonspecific trypsin (31–44 %) and these similarities are based not only on the catalytic domain but also on the other conserved features of the enzymes [7, 8].

The SVTLEs show optimal activity in the alkaline range of pH (7.5–9.0) and at around 37–45 °C temperature (Table 2) and these values are in consistence with many of the earlier reports showing SVTLEs prefer an alkaline pH range for optimum activity.

4 Primary and Secondary Structures Determination

Through the analysis of cDNA, the primary structure of BjussuSP-I from *B. jararacussu* venom was determined [17]. This enzyme is composed of 232 amino acid residues containing 12 half-cysteine residues which form 6 disulfide bonds in enzyme molecule. The enzyme was found to contain all the three essential amino

acids of catalytic triad viz. His40, Ser178 and Asp85 in addition to Asp199, Gly228, and Gly239 which constitute the subsites S1, S2 and S3, respectively [17]. As discussed below, the role of subsites S1 and S2 I is to facilitate the binding of the enzyme with basic residues of substrates (TAME and BApNA), whereas a substitution in subsite S3 may alter the substrate specificity of SVTLEs [7].

Through the theoretical molecular dynamics (MD) simulation programme, the overall structure of the BjussuSP-I was determined which demonstrated the typical fold of a trypsin-like serine protease. The catalytic site residues were found to be located between the 2 six-stranded β-barrels with 6 numbers of disulfide bridges [17]. The loops around the catalytic site 37,60,70,99,148,174 and 218 are thought to be involved in substrate recognition and susceptibility to inhibitors [17]. The authors have suggested that the open disposition of the above described loops in BjussuSP-I is indicative of the fact that the native enzyme may adopt a confirmation suitable for the better interaction with its substrate through the catalytic site triad resulting in higher protease activity displayed by BjussuSP-I [17].

The secondary structure determination using some advanced biophysical methods viz x-ray crystallographic technique has been applied for only a few SVTLEs [18, 19]. A crystal of sexathrombin, a 30 kDa thrombin-like enzyme purified from *Gloydiuss axatilis* venom, was obtained by using hanging-drop vapor diffusion method and it showed diffraction to a resolution limit of 1.43 Å [18]. The crystal belongs to space group C2, with unit-cell parameters a = 97.23, b = 52.21, c = 50.10 Å, β = 96.72°, and the Matthews coefficient (VM) was calculated to be 2.13 Å3 Da-1 with one molecule in the asymmetric unit. It may be expected that this high-resolution structure of sexathrombin would be helpful to understand the differences of this enzyme from thrombin and may assist in thrombolytic drug discovery.

De Oliveira et al. in 2009 [19] have presented the structural model of a thrombin-like enzyme gyroxin by analyzing its local and global stereo chemical properties using VERIFY3D, PROSA, and VADAR and by comparison with other crystallographic structures. The analysis of gyroxin structure through Ramachandran plot indicated 95.3 % of the residues in most favorable region, 3.0 % in additional allowed region, and 1.7 % in disallowed region. The model structure shows that gyroxin is consisting of two domains each containing a six-stranded b-barrel and two short a-helices. The catalytic triad (His57, Asp102, and Ser195) is located at the junction of both barrels and is surrounded by the 37-, 60-, 70-, 99-, 148-, 174-, and 218-loops [19].

5 Substrate Specificity of SVTLEs

Since SVTLEs belong to serine protease family therefore, according to the nomenclature proposed by Schechter and Berger in 1967 [20], the substrate specificity of serine proteases is generally considered on S1/P1 and S1′/P1′ interaction (primary interaction site or S1) in addition to interactions on positions S2/S2′ and S3/S3′ (secondary interaction site or S2). The presence of these subsites in serine proteases

allows them to interact with the basic P1 residue of substrate (S1-P1 interaction) such as TAME and α-N-benzoyl-DL-arginine-*p*-nitroanilide (BApNA), the two most preferred substrates for many of the SVTLEs [7, 8, 11]. However, studies have revealed some structural differences around the active site cleft between SVTLEs and thrombin. For example, thrombin is shown to contain an additional segment of 187–190 in its primary structure around the S1 loop which is lacking in SVTLEs and this facilitates the large substrate side chain access to the catalytic site of thrombin molecule [21, 22]. In addition to S1 and S2 subsites, there is an additional tertiary specificity site (S3) which is occupied by Gly226 is detected in most of the SVTLEs [7]. However, in some of the SVTLEs, the glycine is found to be replaced with alanine and a mutation in this S3 subsite may not affect the overall tertiary structure of the SVTLEs; conversely, a higher specificity for Pro residue instead of Gly and Phe at P2 of the substrate may be expected [7, 8, 23]. Therefore, despite a high degree of similarity in primary structure, a minor variation or substitution in the subsites of the active site of the SVTLEs may lead to differences in the substrate specificity amongst the enzymes [1].

Although a number of different synthetic substrates are hydrolyzed by SVTLEs however, they show a different degree of substrate specificity. For example, TLBan [24], Purpurase [25], TLBm [26], BjussuSP-I [17, 27], AH143 [28] and BJ-48 [16] are reported to show amidolytic activity against BApNA and/or esterase activity against TAME. Nevertheless, some of the SVTLEs for example, Russelobin [14] could not hydrolyze these substrates. The presence of subsites S1 (Asp199/Asp189) and S2 (Gly 228) in SVTLEs play an important role on binding with the substrates possessing basic residues such as TAME and BApNA [17, 29]. Therefore, determinants of substrate recognition and molecular basis of protease specificity may be quite subtle which involves contribution of several factors rather than just a few critical sites in the protease molecule thus justifying the differences in substrate specificity among SVTLEs [1]. The N-linked carbohydrate moieties on SVTLEs are shown to influence their interaction with the macromolecular physiological substrates such as fibrinogen; however, not with small molecules such as TAME or BApNA [7, 14].

Crystallographic studies have shown that three regions (residues 82–99, 192–193 and 215–217) are known substrate binding regions in snake venom serine proteases [30]. During the course of evolution, some mutations are observed in these flanking sequences which may lead to differences in substrate specificity amongst SVTLEs. Russelobin like trypsin is not allosterically regulated by sodium ions and analysis has shown that the proteases containing Pro225 cannot discriminate the monovalent cations resulting in no allosteric regulation [31].

6 Inhibition by Natural and Irreversible Protease Inhibitors

Irreversible inhibition of enzyme activity with classical serine protease inhibitors has been used routinely to determine the presence of serine in the active site. These classical serine protease inhibitors such as PMSF, DFP, AEBSF and benzamidine

significantly inhibit protease activity by irreversibly binding with the serine residue present in the active site of the SVTLEs and by competitive inhibition of arginine reside (P1 specificity reside for SVSPs), respectively [11]. Besides, these inhibitors may even highlight the important structural differences between two functionally related protease molecules. Based on the inhibition study, all the SVTLEs are found as serine proteases. The effect of different inhibitors on enzymatic activity of SVTLEs has been shown in Table 3. They have shown a different degree of susceptibility against the same inhibitor. A computational study of some benzamidine-based inhibitors of SVTLEs has provided valuable information on the topological features of enzymes that determines the divergences in enzyme activity [32].

Interestingly, some of the SVTLEs, for example, BjussuSP-I [17], Bhalternin [33], TLBan [24], and TLBm [26] although are shown as serine proteases; nevertheless, the metalloprotease inhibitors such as EDTA and/or1,10-phenantrolineare reported to inhibit their activity. It may possible that EDTA by chelating a metal ion required in stabilizing the tertiary structure of protein affect the proteolytic activity of these enzymes. However, further studies are necessary to understand the mechanism by which the above inhibitors inhibit the SVTLEs. The SVTLEs contain many intramolecular disulfide bonds and thus supporting the inhibition produced by DTT by reducing the disulfide bonds of SVTLEs [7, 8, 11].

A significant incongruity was observed among the SVTLEs with respect to inhibition of enzyme activity by natural protease inhibitors such as soybean trypsin inhibitor-I, α2-macroglobulin, antithrombin-III and heparin. The amidolytic activity of some of the SVTLEs such as DIII-4 BS3 [34], TLBm [26] and TLBan [24] are inhibited by SBTI however; the same inhibitor cannot inhibit Russelobin [14]. Similarly, common thrombin inhibitor such as heparin and antithrombin-III although do not inhibit most SVTLEs [6] nevertheless, Russelobin [14] and BjussuSP-I [17, 27] have been shown to be inhibited by the above mentioned inhibitors. Two different regions called anion binding exosites (ABE-I and ABE-II) are the key sites on thrombin molecules for binding and subsequent catalysis of its natural substrates and antithrombin-III and heparin bind to these sites thus preventing the catalytic activity of thrombin [7]. Consequently, the SVTLEs lacking these sites are not inhibited by antithrombin-III and/or heparin [7, 35]. It is noteworthy to mention that protease inhibitors of plasma such as α2 MG and antithrombin-III produce only 4–5 % inhibition of enzymatic activity of Russelobin and therefore, there may be a little physiological significance of such inhibition.

7 Significance of Glycosylation on SVTLEs

A major percentage of secretary proteins of the cell undergo post-translation modifications that play a key role in structure-function properties of the proteins or enzymes. Post-translational attachment of carbohydrates to proteins is known as glycosylation which takes place primarily in the endomembrane system. The first step is core glycosylation in endoplasmic reticulum followed by further extensive glycosylation of

protein in the trans-Golgi complex [36]. The carbohydrates are linked to the protein component through glycosidic linkage, which is formed in two ways-(a) N-glycoside linkage through the amide group of asparagine, and (b) O-glycoside linkage in which the OH group of serine, threonine or hydroxylysine is attached with the sugar residues. Most of the SVTLEs are glycosylated in nature and contain variable numbers of N- or O-glycosylation sites in their primary structures [7, 8, 11].

The carbohydrate moieties in SVTLEs, as shown in Table 2, are attached through N-glycosylation but no O-glycosylation was detected. The N-linked carbohydrate content is found to vary from 24 % (TLBan) to 56 % (Russelobin) by mass. Some of the TLEs such as BJ-48, BjussuSP-I, BpSP-I, and TLBan contain 11–14 % sialic acid (N-acetyl neuraminic acid) residue in their carbohydrate structure (Table 2). However, treatment of Russelobin with neuraminidase or O-glycoside does not have any effect on electrophoretic mobility of protein suggesting absence of sialic acid and/or O-linked carbohydrate residues in this TLE from Russell's viper venom [14]. In BjussuSP-I, the N-glycosylation sites are predicted on residues Asn77 and Asn129 [27].

Recently, Luo et al. [37] have determined the structures of N-linked carbohydrate moieties attached with Defibrase, a SVTLEs from *Agikistrodon acutus*, which was achieved through sequential exoglycosidase digestion followed by mass spectroscopic analysis of the digested products. The monosaccharide and sialic acid were detached from the protein molecule through hydrolysis at 110 °C followed by their separation through high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The total carbohydrate content was determined as 19.4 % (w/w) of total protein mass and the most predominant monosaccharide in Defibrase was N-acetyl glucosamine (9.5 %) followed by mannose, sialic acid, galactose, and then fucose suggesting complex oligosaccharides are dominant carbohydrates in Defibrase molecule [37]. The N-linked oligosaccharides were released by deglycosylation with PNGase F, then fluorescent labeled with 2-aminobenzamide followed by fractionation through high performance liquid chromatography. The presence of hybrid and oligomannosidic type oligosaccharides in Defibrase makes it distinct from the N-linked oligosaccharides of other snake venom serine proteases [37].

It has been well documented that glycosylation is required for the proper folding and efficient secretion of many, but no all glycoprotein [36]. Therefore, the importance of glycosylation on protein folding, secretion or for any other purpose must be determined for each individual glycosylated protein. The exact role of glycosylation on SVTLEs is still unclear and many fragmentary and contradictory data are available. Some of the predicted function(s) of glycosylation on SVTLEs are discussed below.

(a) Role of glycosylation in thermostability of protein. Many of the researchers have shown that N-linked carbohydrate residues attached with SVTLEs for example, BJ-48 [16], protect the enzyme from thermal denaturation. However, in a sharp contrast to this observation, Mukherjee and Mackessy [14] observed no difference in thermo stability between native and partially deglycosylated Russelobin, a thrombin-like protease from Russell's viper venom.

(b) Improvement of catalytic resistance against extreme pH and temperature. It has been shown by Costa et al. [33] that glycosylated BpSP-I, a TLE from *Bothrops pauloensis* venom was more resistance against catalytic denaturation in extreme temperature and acid pH as compared to deglycosylated enzyme. However, in case of Russelobin [14] no difference in pH or temperature optima between glycosylated and deglycosylated enzyme was observed

(c) Role of glycosylation against inhibition of enzyme activity. Studies have shown that glycosylated and deglycosylated SVTLEs behave differently against the neutralization of enzyme activity by specific inhibitor. It may be anticipated that some N-linked carbohydrate residue(s) may be involved in steric blockage of a particular macromolecular inhibitor to the enzyme active site. For example, native Russelobin was found to be more resistance against the neutralization of plasma protease inhibitors such as α2MG and antithrombin-III, compared to partially deglycosylated enzyme [14]. Similarly, deglycosylated BJ-48 is more susceptible to inhibition by SBTI but not by infestin, as compared to glycosylated enzyme [16].

(d) Role of glycosylation on alteration of catalytic activity of the enzyme. Many researchers have demonstrated that glycosylation improves the catalytic efficiency of SVTLEs towards the macromolecular substrates such as fibrinogen [7]. In support of this theory, Ana et al. [17, 27] have shown that deglycosylation of native BjussuSP-I resulted in 50 % decrease in fibrinogen clotting activity of the native enzyme. Similarly, partially deglycosylated Russelobin [14] and BpSP-I [38] demonstrated significantly less fibrinogenolytic activity but not amidolytic activity as compared to native, glycosylated enzyme. Zhu et al. [39] have opined that presence of distinct glycan residues linked close to active sites of the serine protease may be responsible for altered catalytic efficiency between glycosylated and deglycosylated enzymes.

However, a contradictory report has been presented by Leme et al. [29]. It was observed that partial removal of N-linked carbohydrate residues from BPA, a thrombin like enzyme from *Bothrops jararaca* resulted in increase in its fibrinogenolytic activity compared to native, glycosylated enzyme thus suggesting partial removal of carbohydrates from BPA may have facilitated its interaction with fibrinogen [29]. However, the mechanism by which partially deglycosylated enzyme better interacted with fibrinogen and the physiological significance of glycosylation on BPA are not clear.

8 Fibrinogenolytic Activity and *In Vitro* Fibrinogen Clotting Activity

It has been shown that The fibrinogen clotting ability of thrombin or SVTLEs is owing to their ability to cleave the Arg16-Lys17 bonds on α and β-chains of fibrinogen and thus converting it to fibrin resulting in release of fibrinopeptide(s) A, B or

AB from fibrinogen [11]. Therefore, on the basis of release of FPA, FPB and/or FPs AB from fibrinogen, the SVTLEs belong to group A, B, or AB, respectively. It is noteworthy to mention that some of the direct-acting fibrinogenolytic serine proteases do not show in vitro fibrinogen clotting activity because they do not liberate fibrinopeptides A and/or B from the fibrinogen molecule [2]. Action of thrombin on fibrinogen resulted in formation of fibrin clot with equivalent release of both the FPs A and B [40]. The majority SVTLEs such as batroxobin, ancord and crotalase, on the other hand, preferentially hydrolyze the Aα-chain of fibrinogen whereas contortrixobin shows release of FP B (see reviews 7, 8, 11).

The fibrinopeptide release pattern by SVTLEs is shown in Table 3. It has been noted that BjussuSP-I, Bhalternin, Purpurase, and Agacutin showed release of only FPA whereas BJ-48 and Fibrinogen-activating enzymes from *Crotalus durissus terrificus* and *C. d. collilineatus* released FPB from fibrinogen. Nevertheless, Russelobin from *D. russelii russelii*, TLE from *Bothrops insularis*, TLBan from *B. andianus* and TLBm from *B. marajoensis* showed preferential release of FPA and slow release of FPB (Table 3). Therefore, they belong to the uncommon Class AB of thrombin-like serine proteases from snake venom. Furthermore, most of the tested SVTLEs demonstrate in vitro plasma clotting activity by virtue of fibrinogen clotting ability without activating the coagulation factor XIII or factor XII [15].

The effect of physiological substrate (fibrinogen) concentration on proteolysis or coagulant activity of some of the SVTLEs was determined and it was noted that with an increase in the fibrinogen concentration beyond a certain level, the fibrinogenolytic or coagulant activity of these enzymes decreased [14, 25]. For example, the fibrinogenolytic activity of Russelobin was inhibited at a fibrinogen concentration higher than 12 μM and authors have reported that fibrinopeptides A and B do not contribute to this inhibition of enzymatic activity [14]. The fibrinogen clotting ability of thrombin-like enzymes such as alborase from *Cryptelytrops albolabris* and purpurase from *Cryptelytrops purpureomaculatus* was also found to be dependent on fibrinogen concentration [1, 25]. At a substrate (fibrinogen) concentration of greater than 5 mg/ml, the fibrinogen clotting time by these enzymes prolonged substantially. The authors could not provide an explanation to these effects and therefore, it may be concluded that further studies are necessary to understand the inhibition of enzyme activity at a higher physiological substrate concentration.

Furthermore, the fibrinogen clotting activities of alborase, purpurase and ancord were also found to vary depending on the source of fibrinogen [1, 25]. For example, the in vitro fibrinogen clotting time of purpurase was observed in this order: cat fibrinogen> human fibrinogen> dog fibrinogen> goat fibrinogen> rabbit fibrinogen (no clot); whereas in case of ancord, the fibrinogen specificity was observed in the descending order as: goat fibrinogen> cat fibrinogen > dog fibrinogen> human fibrinogen > rabbit fibrinogen [1, 25]. Based on the above observations it may be concluded that due to species specific differences in structure of fibrinogen molecules as well as variation at and around the active site, the SVTLEs show differences in hydrolyzing a fibrinogen obtained from the same species [1, 25]. The SVTLEs did not show degradation of fibrin however, the only exception is the Agacutin which for some unexplained reason demonstrated fibrin degradation even at a low dose of 0.025 U/kg [15].

Table 3 Effect of inhibitors and fibrinogenolytic activity of SVTLEs

Name	Inhibitor	Fibrinopeptide release	Plasma clotting activity	References
Russelobin	AEBSF, benzamidine, heparin, a2 MG, antithrombin-III	Both A and B	Yes	[14]
BjussuSP-I	Leupeptin, PMSF, 1,10-phenantroline, heparin	Aα chain	Yes	[17, 27]
Bhalternin	Benzamidine, EDTA (partial)	Aα chain	Yes	[33]
Leucurobin	PMSF, β-mercaptoethanol, benzamidine, SBTI, antibothropic serum	Aα chain, a slow release of FPB after 60 min	nr	[35]
TLBan	PMSF, EDTA and Soybean trypsin inhibitor	Aα-chain, slow release of Bβ-chain	nr	[24]
Purpurase	nr	Aα-chain	nr	[25]
TLBm	PMSF, EDTA and Soybean trypsin inhibitor	Aα-chain, a slow release of fibrinogen Bβ-chain	nr	[26]
Albolabrase	nr	-do-	nr	[41]
Agacutin	PMSF	Aα chain	Yes	[15]
Gyroxin	PMSF, benzamidine	nr	Yes	[42]
Fibrinogen-activating enzyme	Leupeptin, PMSF Heparin (−1,10-Phenathroline (−), EGTA (−), aprotinin (−),	Bβ-chain > Aα chain	Yes	[19]
Coagulant serine protease (AH143)	EDTA (−), Heparin (−) EDTA (−), Heparin (−)	nr		[28]
TLE (DIII-4 BS3)	PMSF, TLCK, Soybean trypsin inhibitor-I	Aα-chain, slow release of fibrinogen Bβ-chain	nr	[34]
BJ-48	Aprotinin, DTT, TLCK, Benzamidine, PMSF TPCK (−), EDTA(−), Hirudin (−)	Bβ-chain, slow Aα-chain	nr	[16]

The SVTLEs viz. BjussuSP-I, Bhalternin, Agacutin, Gyroxin, TLE from *B. asper*, fibrinogen-activating enzyme from *C. d. terrificus* and Russelobin from *D. russelii russelii* in in vitro conditions clotted the platelet poor plasma by virtue of their fibrinogen clotting ability. It was reported that the plasma coagulation mechanism in Agacutin was different from that of thrombin, but similar to that of Reptilase [15].

9 Toxicity and Pathophysiological Effects

Although many of the SVTLEs are purified and biochemically characterized but little attempt has been made to know their lethality and other toxic effects. Table 4 shows a comparison of the lethality and pharmacological properties of some of the SVTLEs reported within the period of 2007 to till date. The SVTLEs such as Agacutin at a dose of 40 U/kg [15] and Russelobin [14] at a dose of 5 mg/kg (i.p) were found to be non-lethal to experimental animals (mice and house lizard). The SVTLEs for example, BjussuSP-I [17, 27], BpSP-I [38], Agacutin [15], and Russelobin [14] did not show any adverse pharmacological effects in treated mice irrespective of the route of administration. However, some of the SVTLEs showed neurological symptoms and/or tissue specific toxicity such as myotoxicity, nephrotoxicity, haemorrhage and edema-induction and the details about their pharmacological properties are mentioned in Table 4.

The neurotoxicological effects of a TLE (gyroxin) isolated from *C. d. terrificus* venom on pup rats were characterized as cellular morphological alterations along with ganglioside composition modifications especially in the hypothalamus, hippocampus and prefrontal cortex parts of the brain [42]. The rats demonstrated pronounced behavioral alterations such as a delayed, maturation of the righting reflex, posture and motor response after the injection of this TLE [42]. Leucurobin, another SVTLEs from *Bothrops leucurus* venom also induced neurotoxic symptom which includes a "gyroxin syndrome" at a dose of 0.143 μg/g mouse [35]. A barrel reaction was observed by Camillo et al. [43] after i.v. injection of gyroxin to mice at a dose of 0.25 μg/g. In a sharp contradiction to this report, Barros et al. [42] did not observe barrel reaction in mice after the administration of gyroxin. However, as reported by Torrent et al. [44] some behavioral effects in mice including initial hyperexcitation and running followed by tachypnea, stretching of posterior limbs and grooming behavior and immobility were observed in gyroxin treated mice [42]. The authors have opined that at the injected dose, gyroxin has no effect on peripheral nervous system [42].

A thrombin-like substance isolated from the venom of *B. insularis* at a dose of 5 μg enhanced the insulin secretion from the isolated pancreatic β-cells of rat about 3.3 fold higher as compared to its respective control [34]. The same enzyme in in vivo condition showed neurotoxicity which was evident from the altered vascular, glomerular and tubular parameters in the isolated rat kidney [34].

Pérez and his colleagues [45] observed that intravenous administration of a coagulant serine proteinase from *Bothrops asper* at a dose higher than 5 μg induced a series of behavioral changes in mice within the first 2–3 min after injection. The prominent features were loss of the righting reflex, opisthotonus, and intermittent rotations over the long axis of the body. These effects persisted during approximately 10 min, after which the mice apparently recovered from this gyroxin like syndrome.

Table 4 Lethality, pathophysiology and pharmacological properties of SVTLEs reported in literature from 2007 (nr: not reported)

SVTLE	Lethality	Toxicity and pharmacological properties	References
Gyroxin like serine protease	nr	Cellular morphological change and ganglioside level modification in brain, Abnormal motor activities (neurotoxin symptom)	[44]
Leucurobin	nr	Gyroxin syndrome (neurotoxin syndrome)	[35]
Thrombin-like substance	nr	Nephrotoxicity, enhances insulin secretion from pancreatic cells	[34]
BjussuSP-I	nr	Does not cause platelet aggregation, non-haemorrhagic, no organ specific in vivo toxicity, induces moderate edema in mice, neurotoxic symptoms not detected	[17, 27]
TL serine-protease	nr	Behavioral change in mice, loss of the righting reflex, opisthotonus, and intermittent rotations over the long axis of the body during first 10 min of injection at a dose over 5 μg	[44]
BpSP-I	nr	No in vivo toxicity, edema-induction, haemorrhagic activity and neurotoxicity in experimental mice	[38]
Agacutin	Non-lethal at a dose of 40 U/kg	No haemorrhagic activity, neurotoxicity and behavioural changes in mice	[15]
Bhalternin	nr	In vivo organ specific toxicity, haemorrhagic activity	[33]
TLBm	nr	Causes platelet aggregation	[26]
Gyroxin	Non-lethal at dose of 10 μg	No effect on peripheral nervous system but abnormal behavioural effects on mice	[42]
Russelobin	Non-lethal up to a dose of 5 mg/kg (i.p)	No in vitro toxicity against mammalian cells, no in vivo toxicity, no neurotic symptom in mice, no behavioural change or abnormalities in mice	[14]

10 Bioprospecting of SVTLEs for Their Therapeutic Application

During the last few decades many of the SVTLEs are well characterized in terms of their biochemical properties and biological activities; however, only a few of them such as ancord, Defibrase and Reptilase have been explored commercially either as a therapeutic agent to treat various cardiovascular disorders or as a diagnostic reagent for the detection of disfibrinogenemia [11]. The Reptilase is used for quantitative determination of fibrinogen in plasma of patients undergoing heparin treatment [7].

The in vivo defibrinogenating activity is described for almost all the SVTLEs. The defibrinogenating activity was evident from the increase in the in vitro coagulation time of blood obtained from SVTLEs treated mice as compared to control group of mice [14]. This result corroborates well with the dose-dependent decrease in plasma fibrinogen concentration in treated mice as compared to control group of mice suggesting incoagulable blood was due to decrease in fibrinogen content of plasma [14].

Leme and his colleagues [29] have demonstrated the in vivo therapeutic application of *Bothrops* Protease A (BPA) in prevention of thrombus formation in rats. Thirty minutes before the induction of thrombus formation in the jugular vein and vena cava, the groups of rats were treated with 250 μg/kg BPA or 300 IU/kg heparin. The administration of BPA completely abolished thrombus formation in both the models of rats which may be attributed to in vivo defibrinogenating activity of BPA [29]. The in vivo defibrinogenating activity of Agacutin was also demonstrated through analysis of mouse tail bleeding time [15]. The *i.v.* administration of Agacutin in rabbit exerted dose-dependent decrease in the fibrinogen content of plasma although it returned back to normal level within 12 h after the injection suggesting therapeutic potential of Agacutin in the treatment of cardiovascular disorders [15].

The evaluation of in vivo defibrinogenating potential of Russelobin, a non-toxic thrombin-like serine protease first reported from Russell's viper venom also showed dose-dependent decrease in fibrinogen content of the plasma of treated mice as compared to control group of mice. Therefore, the future therapeutic application of Russelobin for the treatment of hyperfibrinogenemia related disorder seems to be highly promising [14]. Moreover, leucurobin from *Bothrops leucurus* is not inhibited by heparin and therefore, this thrombin-like enzyme may be considered as a potential candidate for use as a diagnostic reagent in quantitative determination of fibrinogen content of the plasma of patients undergoing heparin treatment as well as for routine assay of blood coagulation factors [35].

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Macrocyclic Protease Inhibitors Constrained into a β-Strand Geometry

Ashok D. Pehere and Andrew D. Abell

Abstract Proteases almost universally bind to their inhibitors and substrates in such a way that the component amino acid backbone is constrained into an extended β-strand conformation. One important general approach to inhibitor design, as discussed here, is to pre-organise the structure into this conformation. This can lead to improved potency, biostability, resistance to proteolytic degradation, and hence therapeutic potential. Here we present an overview of some different synthetic approaches that have been employed for introducing such a macrocycle, with reference to selected examples. We also briefly discuss some representative naturally occurring examples of such macrocyclic protease inhibitors.

Keywords Macrocycles protease inhibitors • Synthesis • β-Strand • Click chemistry • Ring closing methata thesis • Peptidomimetics

1 Introduction

The six known classes of proteases play a major role in the regulation of key biological processes and as such their inhibition provides a basis of therapeutics for treating a wide range of diseases [1, 2]. These include cancer [3, 4], cataract [5, 6], HIV [7, 8] and neurological diseases such as Alzheimer's [9]. There are many classes of protease inhibitor, however the substrate recognition sequences of the enzyme is invariably incorporated into the design, where each amino acid R group (designated P) within the inhibitor (or substrate) binds with a complementary binding pocket (designated S) in the protease active site [10, 11]. With few exception,

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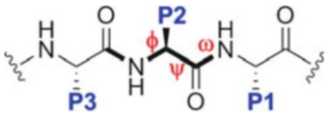

Fig. 1 Peptide β-strand backbone with torsional angles Φ, Ψ, and ω [12]

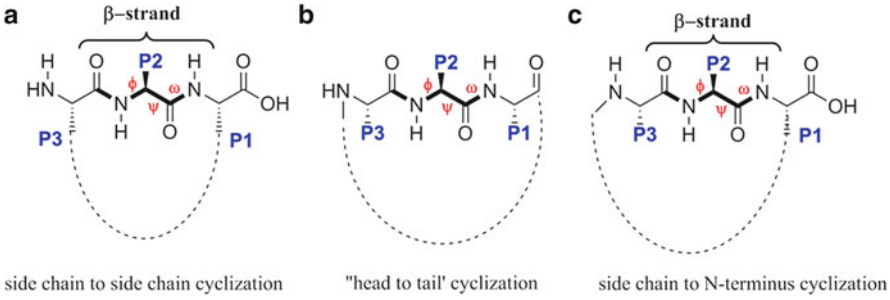

Fig. 2 Different approaches for peptide cyclization

the associated interactions between the two constrain the backbone of the inhibitor into a β-strand geometry that is defined by Φ, Ψ and ω bond angles as depicted in Fig. 1 [12]. A study of more than 1,500 X-ray structures in the Protein Data Bank confirms that proteases do almost universally bind ligands in this geometry [13]. The introduction of an appropriate macrocycle into the backbone of an inhibitor can effectively pre-organize it into such a β-strand geometry and thus reduce the entropy of binding to its target protease. This can also lead to enhanced stability towards proteolytic cleavage, while also providing an opportunity for rational inhibitor design to improve potency, selectivity, and hence therapeutic potential [12, 14–18]. Such an approach to inhibitor design is the subject of this review, with reference to selected examples. While there are natural examples of macrocyclic protease inhibitors most are of synthetic origin.

2 Synthetic Macrocyclic Protease Inhibitors

A number of general classes of macrocyclic peptidomimetics are known that constrain a peptide backbone into a specific geometry, for example those resulting from side chain to side chain cyclization (principally P1 to P3, Fig. 2a), N-terminus to C-terminus cyclization (Fig. 2b, these structure are not necessarily constrained into β-strand and as such are not discussed in this review), and less commonly side chain to *N*-terminus cyclization (Fig. 2c). The choice of ring size is important and while small rings significantly reduce conformational flexibility they do not generally define a β-strand geometry. In comparison, larger rings are usually too conformationally mobile to be of general use. The chemical makeup of the tether within the macrocycle is also critical, where the known recognition sequences of the target

protease must be incorporated into the design. Some of this functionality, such as an aryl group, has the added advantage of further reducing conformational mobility and hence helping to define the overall geometry. The macrocycles of these inhibitors are incorporated using a number of synthetic strategies, including lactamisation (Fig. 3a, b), [12, 19, 20], alkylation (Fig. 3c) [12, 19, 21], ring closing metathesis (RCM) (Fig. 3d) [22–27], and most recently azide-alkyne cycloaddition (Fig. 3e) [28, 29].

2.1 *Macrocyclic Protease Inhibitors Formed by Alkylation and Lactamisation*

A number of potent and selective macrocyclic HIV-1 inhibitors (e.g. **5**) have been prepared by alkylation of the tyrosine aromatic ring of **2** to link the P1 residue to the N-terminus as shown for **3** in Fig. 4 [12, 19]. The incorporation of a hydroxyethylamine isostere into the C-terminus of **3** then gave the active inhibitor **5**. The key starting dipeptide **2** was prepared by N-acylation of the dipeptide **1** as shown. This general approach has been used to prepare a number of other macrocyclic HIV-1 inhibitors, including **6** and **7** as shown in Fig. 4. Inhibitor **7** is particularly interesting in that it contains two macrocycles, one linking P1 to the N-terminus and the other P1' to the C-terminus. All three macrocycles are potent inhibitors the HIV protease with Ki values of 1.7 nM (**5**), 0.6 nM (**6**), and 5 nM (**7**). More recently, we reported a preparation of potent macrocyclic calpain inhibitors of type **13** in which the constituent P1 and P2 residues are linked by lactamisation, see Fig. 5. The synthetic sequence began with *N*-alkylation of the 1,4 disubstituted 1H-imidazole of **8** on treatment with NaH, followed by reaction of the resulting anion with iodide **9** to give the key intermediate **10** [20]. The Boc and ^{t}Bu ester protecting groups of **10** were simultaneously removed on treatment with TFA in CH_2Cl_2 to give **11**, which was cyclized on treatment with EDCI and HOAt to give the key macrocycle **12**. Introduction of the C-terminal aldehyde then gave the active 15-membered macrocycle **13** which had an IC_{50} of 238 nM against calpain II. For comparison, the analogue with the imidazole at the alternative P1 position (**14**) is significantly less active with an IC_{50} of 1,000 nM [20]. Interestingly molecular modelling shows that the backbone of **13** adopts a β-strand geometry while that of **14** does not [20].

2.2 *Macrocyclic Protease Inhibitors Prepared by Ring Closing Metathesis (RCM)*

Ring-closing metathesis (RCM) has found much use in the preparation of conformationally constrained amino acids [30], peptides [31], and macrocyclic peptidomimetics [22, 23, 25, 26, 32]. This strategy has been used to prepare β-strand constrained macrocyclic protease inhibitors of plasmepsin [33], β-secretase (BACE)

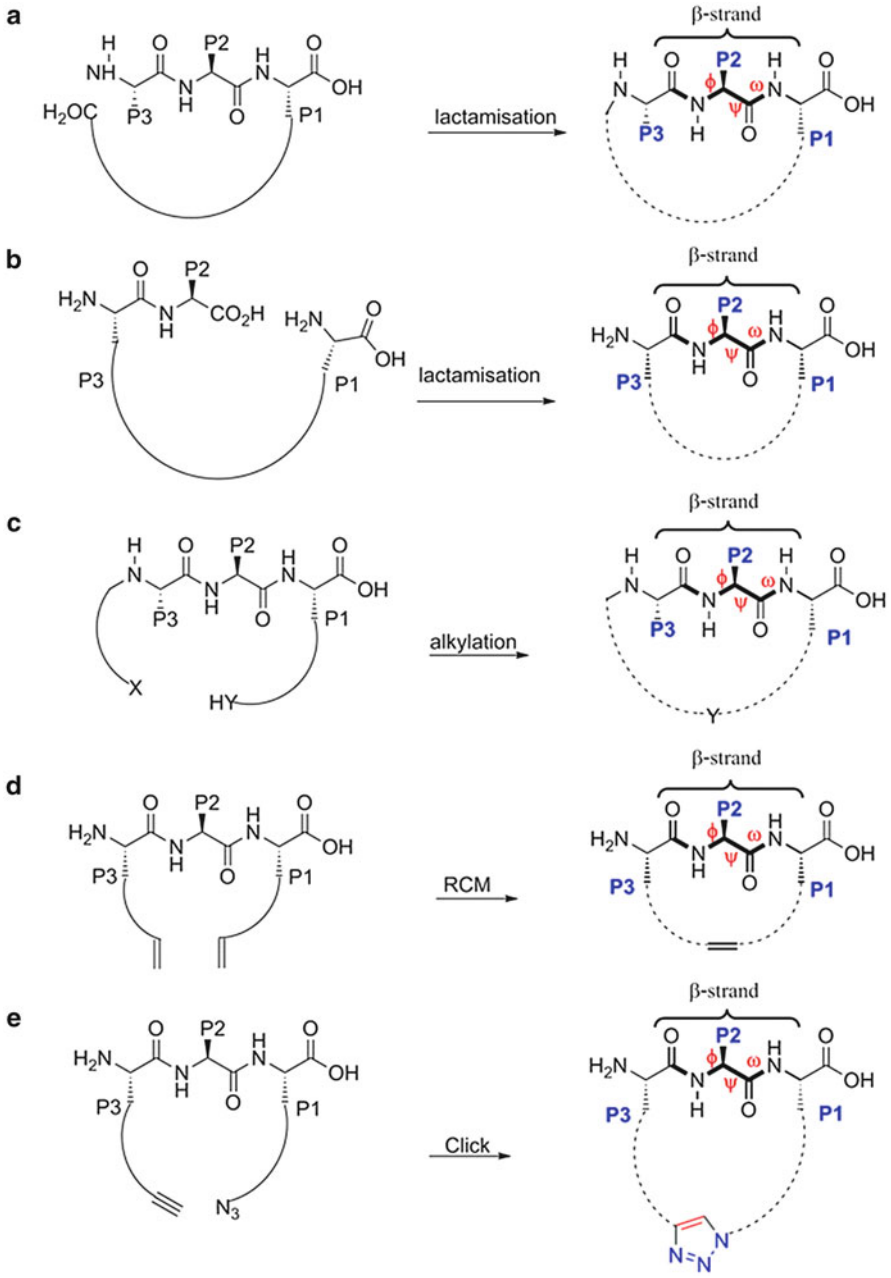

Fig. 3 Macrocycle formation by (**a** and **b**) lactamisation, (**c**) alkylation, (**d**) ring closing: metathesis (RCM), and (**e**) azide-alkyne cycloaddition

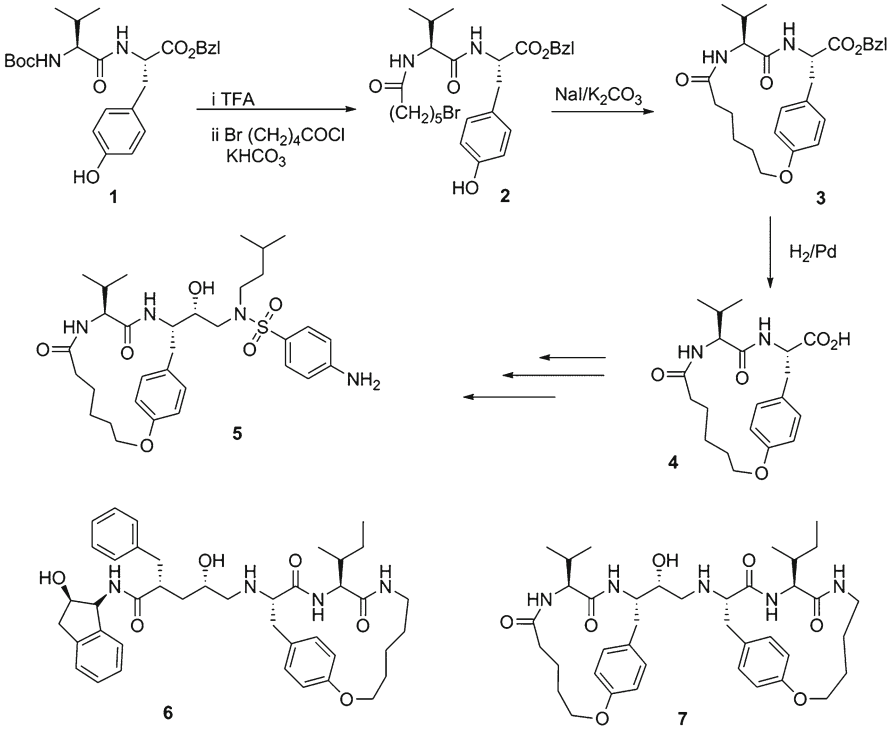

Fig. 4 Macrocyclic HIV-1 protease inhibitors prepared by alkylation

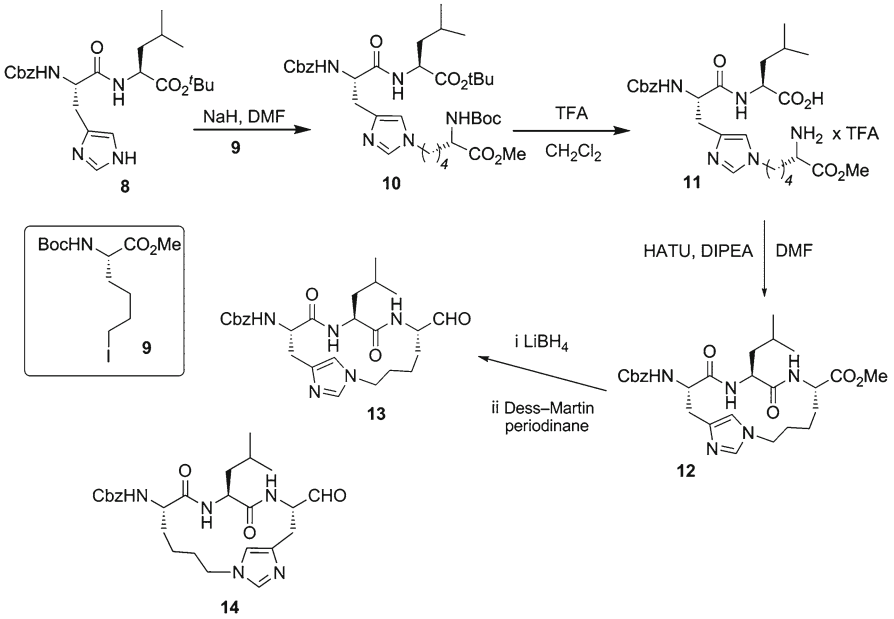

Fig. 5 Macrocyclic inhibitors prepared by lactamisation

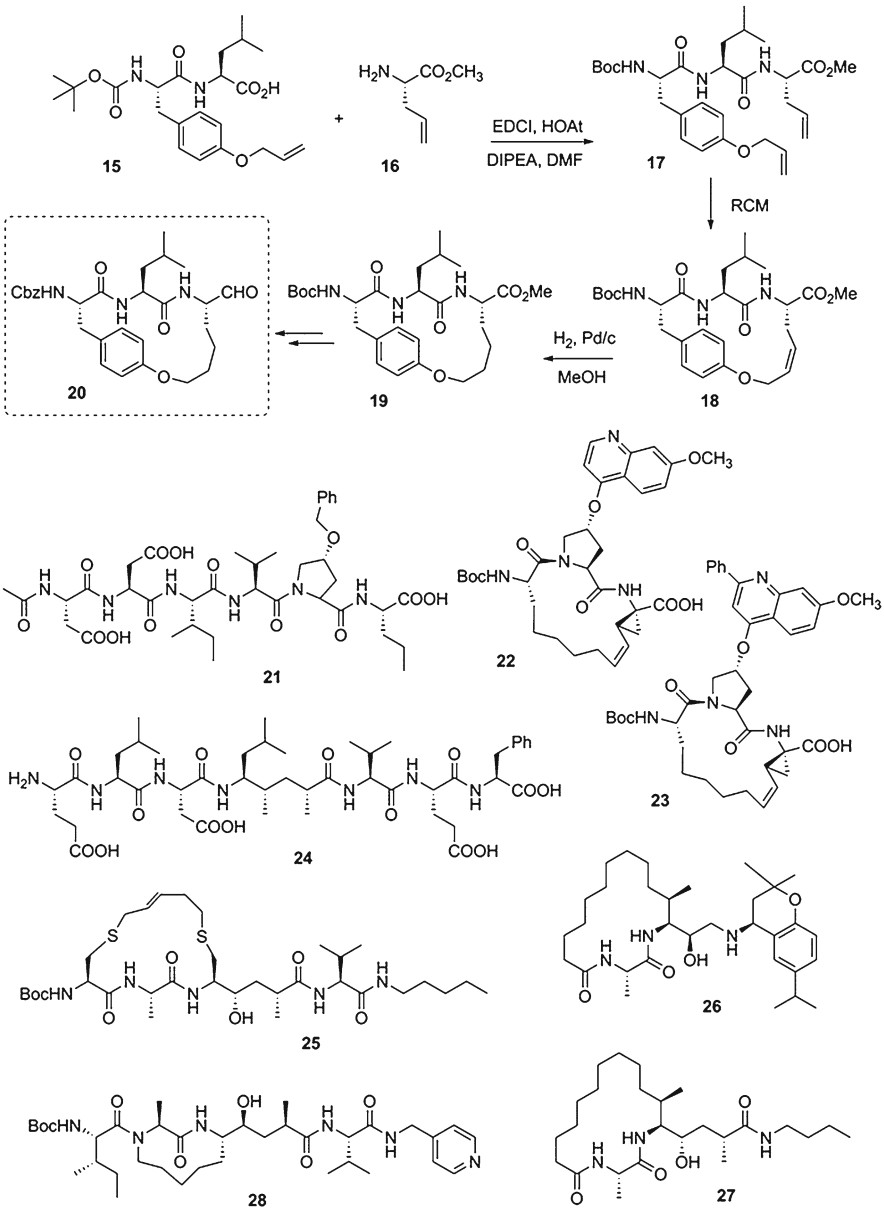

Fig. 6 Examples of macrocyclic inhibitors prepared by RCM and other key structures

[34, 35], HCV NS3 [25, 36], and calpain [22] as shown in Fig. 6 for the representative example **20** (CAT811). The key step in the synthesis of **20** involves treatment of the diene **17** with Grubbs second generation catalyst to give **18**, the double bond of

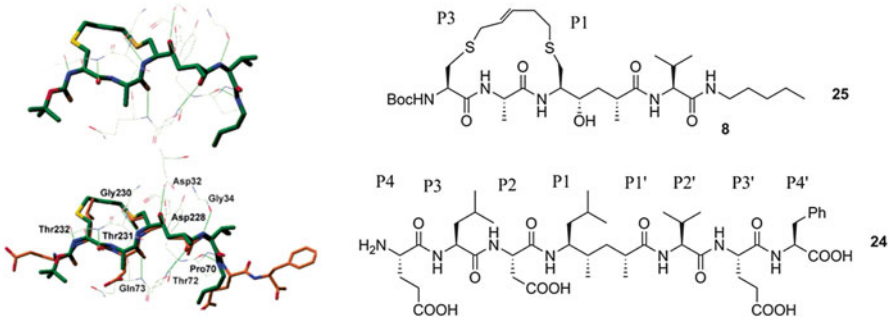

Fig. 7 X-ray structure of macrocycle **25** (*dark green*)-BACE complex (*top*) and overlay of the X-ray structures of BACE complexes with macrocycle **25** (*dark green*) and acyclic **24** (*orange*) (*bottom*) [26]

which was hydrogenated on treatment with H_2 and palladium on carbon to give **19**. The final steps in the synthesis required conversion of the N-terminal Boc group to Cbz and reduction of the C-terminal ester to give the active aldehyde **20**. The macrocycle here links the P1 and P3 amino acid R groups of the backbone to define the required β-strand geometry [22]. The 17-membered tyrosine based macrocycle **20** (**CAT811**) is a potent inhibitor of calpain II ($IC_{50} = 30$ nM) and shows significant promise in the treatment of calpain induced cataract [22]. A conformational search [22, 24] shows that **20** does indeed exhibit a high percentage of desired β-strand conformers [22, 24]. Docking studies with calpain also revealed that **20** adopts three key hydrogen bonds with Gly271 and Gly208 of the active site of calpain to stabilise the optimum β-strand conformation of the peptide backbone chain [22].

Other macrocyclic peptidomimetics have been prepared using a similar RCM-based strategy, e.g. see **22**, **23** and **25**–**27** in Fig. 6. The 15-membered macrocycle **22** is a potent inhibitor of the HCV NS3 serine protease with an IC_{50} value of 24 nM [25] By comparison, the acyclic analogue (**21**) proved to be significantly less active, with an IC_{50} of 400 nM. Interestingly, NMR and molecular modeling studies show that both structures bind to the protease in an extended β-strand conformation. The inclusion of an additional phenyl substituent on the P2 proline, as in **23**, enhanced potency with an observed IC_{50} of 11 nM [25].

The P1 and P3 linked macrocyclic peptidomimetic **25** is a potent aspartic protease BACE inhibitor with an IC_{50} of 156 nM [26]. An overlay of the X-ray crystal structures of the macrocyclic inhibitor **25** and the linear peptide **24** is shown in (Fig. 7). The backbones of both adopt an extended β-strand conformation in the active site of the protease. Crucial hydrogen-bonds observed in the complex of the acyclic inhibitor **24** with active site of the protease (particularly with Gly34, Pro70, Thr72, Gln73, Gly230, and Thr232) were also observed with macrocycle **25**. The macrocycle of **25** therefore stabilizes a bioactive β-strand conformation that is known to favour active-site binding. The final macrocycles in this series (**26**–**28**) are inhibitors of β-secretase (BACE-1). Inhibitor **27** shows modest activity

(IC_{50} = 0.15 μM) while the 16-membered macrocycle **26**, with additional functionality at the C-terminus, has much improved potency with an IC_{50} of 27 nM. The macrocycle of both inhibitors links the P1 residue to the N-terminus [32, 37]. The final macrocycle in this series (**28**) contains an unusually small macrocycle that links the P1 residue to the P3 nitrogen. Somewhat surprisingly this is also a good inhibitor of β-secretase (BACE-1) with an IC_{50} of 650 nM.

2.3 Macrocyclic Protease Inhibitors Prepared by Azide-Alkyne Cycloadditions

Huisgen cycloaddition of an alkyne with an azide has found wide use in the preparation of bio-conjugates that possess a range of properties and functions [38]. Of particular interest in the context of this review is the use of this reaction to prepare cyclic peptidomimetics, where the constituent macrocycle defines a β-strand geometry as in **35** in Fig. 8 [28]. The synthesis of **35** involves treatment of the key intermediate azido alkyne **33** with Cu(I)Br in CH_2Cl_2 to give the triazole-containing macrocycle **34**, the primary alcohol of which was then oxidized to give the active inhibitor (see Fig. 8). Here again the macrocyclic precursor (**33**) is readily prepared by standard peptide synthesis as outlined in Fig. 8. A range of macrocyclic protease inhibitors (e.g. see **35**–**42**, Fig. 8) has been prepared using this approach, with their backbone geometries having been determined and defined by detailed NMR analysis [28, 29]. All these macrocycles **35**–**42** displayed $^3J_{NHC\alpha H}$ coupling constants >8 Hz, which suggests a Φ torsion angle of ≈−120° and hence a β-strand geometry for the associated backbone [28, 29, 39]. Docking and X-ray crystallographic studies were also consistent with an assignment of this geometry [29, 39].

The macrocyclic aldehydes **35**–**42** were assayed against a range of different proteases [39]. Macrocycles **35**–**39** were particularly potent against cathepin S (IC_{50} < 5 nM), while **35**, **36** and **37** were also highly active against calpain II (IC_{50} values of 137 nM, 97 nM and 89 nM, respectively). In addition, **35**–**38** were weakly active against the chymotrypsin-like activity of the proteasome (IC_{50} < 1 μM), while being essentially inactive against the other proteasome activities (i.e. trypsin-like and caspase-like). The macrocycles **35**–**37** were significantly more active against calpain II and cathepsin L than their acyclic analogues, again suggesting that constraint into a β-strand geometry favours active site binding [39]. Interestingly the acyclic analogues were generally more active against the chymotrypsin-like activity of the proteasome. This observation may reflect the fact that there is some suggestion that the proteasome does not favour binding in a classic β-strand geometry: unlike other proteases the P2 group of the inhibitor does not seem to form important contacts with the active site. Interestingly, the 1,5-disubstituted triazole **39** retains potency against calpain II, cathepsin S and the 26S proteasome, but has reduced activity against cathepsin L. This suggests one possible avenue for influencing selectivity for one protease over another.

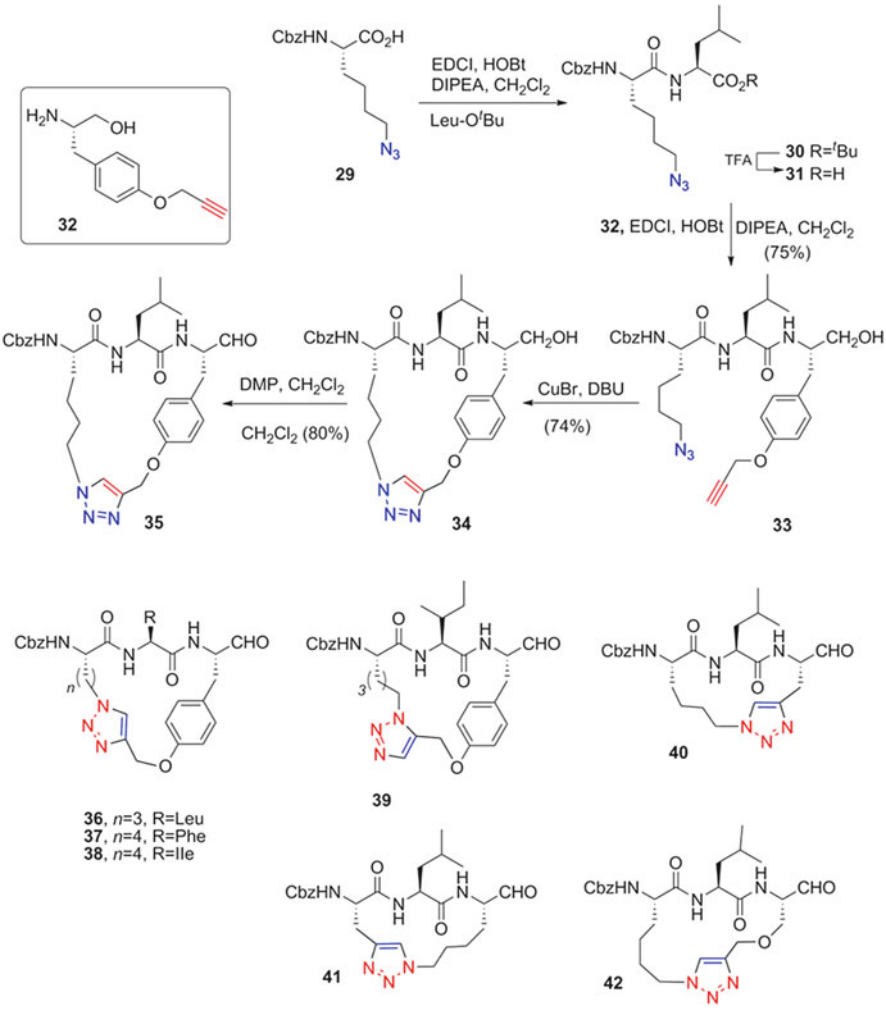

Fig. 8 Macrocyclic inhibitors prepared by azide-alkyne cycloaddition

3 Naturally Occurring Macrocyclic Protease Inhibitors

There are a few examples of naturally occurring macrocyclic protease inhibitors that are constrained into a β-strand geometry. Some representative examples, where the P1 and P3 residues of the component backbone are linked with a diarylether tether, are shown in Fig. 9. The 17-membered macrocycles **43** (QF4949-IV) and **44** (K-13) are potent inhibitors of both aminopeptidase B and acetylcholinesterase (ACE) [40, 41]. Molecular modeling, NMR and X-ray analysis revealed that the 17-membered

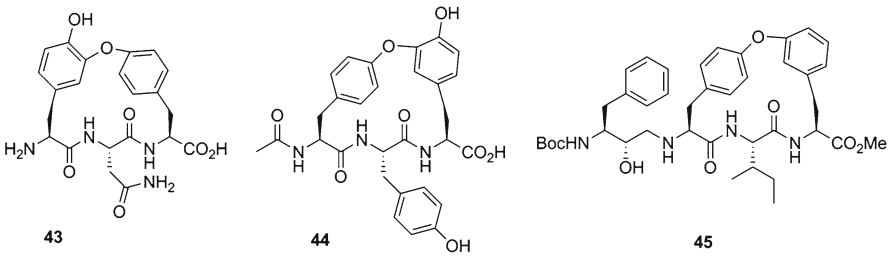

Fig. 9 Diarylether-based Macrocyclic protease inhibitors

macrocycles of **43** and **44** do indeed define a β-strand conformation [42, 43]. These macrocycles inspired the subsequent design of several β-strand based HIV protease inhibitors that contain a hydroxyethylamine isostere, e.g. **45** [43].

4 Conclusion

In this review, we present some representative examples of macrocyclic protease inhibitors that are constrained into a particular geometry, typically a β-strand, which favours active site binding. The component macrocycles are incorporated using a number of general methods; particularly alkylation, lactamisation, ring closing metathesis and azide-alkyne cycloaddition. Naturally occurring examples of such macrocyclic protease inhibitors are also known and these have inspired the subsequent design of other macrocycles that target different proteases, particularly the HIV proteases as discussed here. The macrocyclic component of all these synthetic and naturally occurring inhibitors invariably links either the P1 and P3 amino acid R groups, amino acid R group to the N-terminus, or less commonly an amino acid R group to the C-terminus. Much work has been done to define the geometries of these macrocyclic protease inhibitors and to begin to exploit their potential against a range of different proteases and in a number of important therapeutic indications.

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Part II
Involvement of Proteases in Disease Processes

Regulation of MT1-MMP Activation and Its Relevance to Disease Processes

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Abstract Matrix metalloproteases (MMPs) are members of zinc-dependent enzymes involved in the degradation of extracellular matrix (ECM) components. Among these enzymes, membrane-type 1 MMP (MT1-MMP) has been established as a vital enzyme in pericellular proteolysis of the ECM macromolecules and shown to be involved in regulating different cellular and tissue characteristics and function in normal and pathological conditions. The enzyme is also important for skeletal development, cancer invasion, growth, neovessel formation, cell invasion as well as various signal transduction events. In this review, we summarize some information about regulation of MT1-MMP expression and activity and the recent knowledge of participation of MT1-MMP in different physiological and pathological events.

Keywords Extracellular matrix • Matrix metalloprotease • Membrane type1 matrix metalloprotease • Signal transduction • Angiogenesis

1 Introduction

Proteases are important in various physiological and pathological processes. Based on the preference of cleavage of peptide bond in substrates, they can be classified as exopeptidase (terminal peptide bond) or endopeptidase (internal peptide bond). Most endopeptidases are classified as serine, cysteine, aspartic or metalloproteases based on their catalytic mechanism and inhibitor sensitivities. Matrix metalloproteases (MMP) are multifunctional zinc-dependent endopeptidases that can degrade a variety of extracellular matrix (ECM) components, like collagens, laminin,

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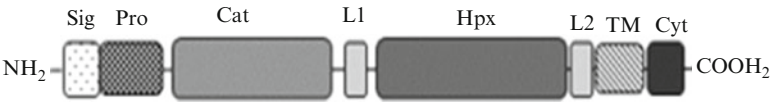

Fig. 1 Domain arrangements of MT1-MMP. MT1-MMP is synthesized with a signal peptide (Sig) for its trafficking to membrane, a prodomian (Pro) for its latency, a catalytic domain (Cat) with catalytic zinc atom (Zn) for proteolytic activity, a hinge region (L1, linker 1) which provides better flexibility of catalytic and Hpx domains, a hemopexin domain (Hpx) which interacts with cell surface molecules like CD44 and homodimer formation, a stalk (L2, linker 2) region, a type-1 transmembrane domain (TM), and a cytoplasmic tail (Cyt), important for clathrin-mediated internalization of MT1-MMP, recycling and palmitoylation

fibronectin, vitronectin, aggrecan, enactin, versican, perlecan, tenascin and elastin [1]. The MMP family of enzymes consists of 24 zinc-dependent endopeptidases in humans. MMPs are secreted as soluble or membrane type-MMPs (MT-MMPs), which are anchored to the cell surface through transmembrane domain or glycosylphosphatidylinositol (GPI) linker.

MT-MMPs are type 1 transmembrane proteins with a single membrane spanning domain and are synthesized as inactive precursor. Unlike other MMP family members, the MT-MMPs possess an additional C-terminal domain that helps tether them to the membranes of the cell surface and thereby rendering them as an important effector in pericellular proteolysis. Nevertheless, MT-MMPs have similar domain organization to all other MMP family members including a signal peptide, a prodomain, a catalytic domain, a hinge region and a hemopexin domain [2]. Among the six MT-MMPs identified to date, MT1, MT2, MT3 and MT5 have a type I transmembrane domain; while MT4 and MT6-MMP are tethered to the plasma membrane by a GPI anchor [3, 4]. MT1-MMP after its synthesis contains the following regions, a signal sequence, a propeptide, a catalytic domain, a hinge region (linker-1), a hemopexin (Hpx) domain, a stalk region (linker-2), a transmembrane domain, and a cytoplasmic tail (Fig. 1) [2].

The MT-MMP family members were first identified as activators of soluble MMPs and was originally thought to exhibit a single function as a membrane activator of soluble MMPs, including MMP-2 [5, 6] and MMP-13 [7]; but later shown to be able to degrade other ECM components such as fibrillar collagen, laminin-1, laminin-5, aggrecan, fibronectin and fibrin (Fig. 2) [2, 8]. Furthermore, they can proteolytically liberate and modify growth factors and cytokines. In addition to its role in extracellular proteolysis, MT1-MMP has also been shown to be involved in processing of intracellular substrates such as tissue transglutaminase, fibronectin, pericentrin, pro-αv integrin and focal adhesion kinase [2, 9–11].

Like all other MMPs, MT1-MMP is synthesized as a latent proenzyme and requires N-terminal processing to be an active mature membrane bound enzyme. MT1-MMP catalytic activity can be inhibited by tissue inhibitor of matrix metaloproteases (strongly by TIMP-2, -3 and -4, but poorly inhibited by TIMP-1) [12] as well as by a GPI anchored glycoprotein RECK (reversion-inducing cysteine-rich protein with Kazal motifs) [13]. Except MT1-MMP, mutations in MMP genes or

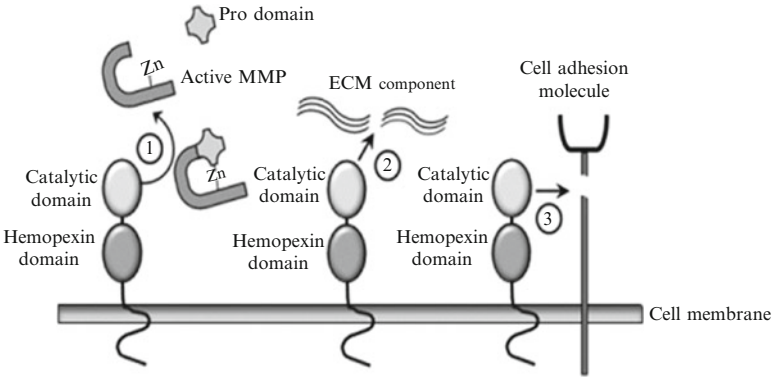

Fig. 2 Proteolysis and signal transduction by MT1-MMP. MT1-MMP mediates activation of proMMPs including proMMP-2 and proMMP-13. The catalytic domain of MT1-MMP cleaves the pro domain of proMMPs and produces the active MMP. MT1-MMP directly degrades ECM components like collagen I, II, III, vitronectin, fibronectin etc. as shown in the figure. MT1-MMP also cleaves cell adhesion molecules like CD44 or integrin. The hemopexin domain helps attach the receptor molecule with MT1-MMP itself. MT1-proteolytically inactivates ADAM9 and cleaves Notch ligand in B cell in signal transduction processes. MT1-MMP is also engaged in ERK activation and Src kinase activation, which seems to occur via its cytoplasmic domain

gene knockout mice do not show lethality or any easily identifiable phenotype, which implicates the redundancy in function of MMPs. On the other hand, MT1-MMP knockout mice develop including dwarfism, osteopenia, progressive fibrosis, arthritis and skeletal dysplasia owing to defect in degradation of type I collagen [14]. Recent report also indicates that MT1-MMP may function as a fibrinolytic enzyme in absence of plasmin and mediates pericellular proteolysis in angiogenesis [15]. MT1-MMP expression has been reported to be correlated with the malignancy of different kind of tumours [16] as well as an important effector of cell invasion and migration [17, 18].

2 Regulation of MT1-MMP Activity

2.1 Proteolytic Processing of MT1-MMP Molecule

MT1-MMP or the MMP-14 gene is located on human chromosome 14q11–q12 [19]. The enzyme has been shown to be processed by furin-like proprotein convertases which recognizes the unique amino-acid sequence (RRKR) located between the pro and catalytic domains during secretion in the Golgi bodies [20]. In addition, MT-MMPs have a unique regulatory mechanism in which the active enzyme undergoes a series of processing steps, either by autocatalytic processes [21–23] or

mediated by other proteases [24], which modulate the activity of the enzyme at the cell surface as well as at the pericellular space. MT1-MMP has been shown to be auto catalytically processed at the cell surface to an inactive membrane-tethered ~44-kDa form lacking the entire catalytic domain [23]. This ~44-kDa species contains the hemopexin domain and may play a role in regulating activity of the mature enzyme. This processing is inhibited by TIMP-2, TIMP-4 and by some synthetic MMP inhibitors [24, 25]. Inhibition of MT1-MMP processing results in accumulation of the active enzyme on the cell surface, and therefore, net MT1-MMP dependent proteolysis is enhanced. In fact, several reports have documented that, under certain conditions, inhibition of MT1-MMP autocatalysis by synthetic MMP inhibitors enhances activation of proMMP-2 by MT1-MMP in presence of TIMP-2 [25, 26]. Therefore, while the presence of the inhibitors induces accumulation of the active enzyme on the cell surface, their absence or diminished levels hasten autolysis of the enzyme. In some cells, the whole ectodomain including the catalytic domain of MT1-MMP has been shown to be shed [27–29]. The shedding releases functional MT1-MMP from the cell surface. Catalytic activity of this shed MT1-MMP would be lost in spite of its presence in the extracellular milieu, since the enzyme needs to be membrane-anchored to exert its biological action.

2.2 Intracellular Trafficking

MT1-MMP is shown to be regulated at the transcriptional as well as post transcriptional levels by well organized and versatile mechanisms. Degradation of collagen on the cell surface by membrane-bound MT1-MMP is controlled both spatially and temporally because the enzyme is unable to bind to the substrate by itself, and the half-life of the enzyme is less than half an hour on the cell surface because it is constantly endocytosed via clathrin-dependent and independent pathways. Internalization via clathrin-coated pits and through clathrin-independent caveolae vesicles have been shown to be an important mechanism for regulation of MT1-MMP activity [30–32]. Internalization of MT1-MMP via clathrin-coated pits is mediated via its cytoplasmic tail which provides a means to control active pool of MT1-MMP on the cell surface. However, the internalization process seems to be essential to promote cell migration [33]. Recent study has documented that MT1-MMP is internalized in clathrin-coated vesicles to early endosomes through a dynamin-dependent process [31]. Apart from clathrin-dependent endocytosis of MT1-MMP, another way of trafficking the enzyme to the intracellular compartment is via caveolae microdomains. Caveolae are cholesterol enriched plasma membrane invagination involved in internalization of receptors, recycling of molecules via the Golgi bodies and signal transduction pathways, and found in many but not all cell types. Despite the growing evidence of caveolae mediated intracellular trafficking of MT1-MMP, little is known about the molecular mechanisms that control the uptake of enzyme molecules into the cell. Previous investigation reported that a part of internalized MT1-MMP is transported to the CD63 positive lysosomes for degradation [34]. CD63, a well

established component of late endosomal and lysosomal membranes, interacts with MT1-MMP directly via the N-terminus of CD63 and the hemopexin domain of MT1-MMP [34]. CD63 accelerates internalization and lysosomal degradation of MT1-MMP, requiring both its hemopexin and cytoplasmic domains [34]. Thus, CD63 seems to be an important mediator of MT1-MMP trafficking into the cells. Indeed, previous reports have documented that CD63 and CD151, a family of tetraspanins (membrane spanning molecules containing four trans membrane domains) inhibit MT1-MMP dependent MMP-2 activation [34, 35].

2.3 Transcriptional Regulation of MT1-MMP Gene

The MT1-MMP gene consists of ten exons and nine introns and there are four major and several minor transcription start sites in the promoter region of the MT1-MMP gene [36]. The binding sites for transcription factors Egr-1 and Sp-1 lies close to the multiple transcription start sites and Sp-1 binding site is crucial for the expression of the MT1-MMP gene [37]. In the upstream position of Egr-1 and Sp-1 binding sites, there is a v-src-responsive enhancer element that mediates enhanced expression of MT1-MMP gene in v-Src transformed Madin-Darby canine kidney cells [37]. In the 5′ flanking region of MT1-MMP gene there is Tcf4-binding promoter sequence that is responsible for the expression of the gene in colon carcinoma cells, and motif is connected directly with the β-catenin/Tcf4 complex [38]. β-catenin, on the other hand is negatively regulated by wnt signaling induced by APC (adenomatous polyposis coli) gene. Alteration of this regulation by mutations of APC gene increases the β-catenin level in cytoplasm and promotes its translocation into the nucleus thereby activating the MT1-MMP gene. The promoter region of the human MT1-MMP gene also contains consensus binding sites for nuclear factor kappa B (NF-kB), which may be activated by intracellular signal transducers, for example, protein kinase C alpha (PKC-α) [39, 40].

3 MT1-MMP Stimulates Cell Motility

MT1-MMP promotes cell migration and invasion by degrading the ECM barrier. Among the ECM components, collagen is most abundant in humans and forms the structural framework basement membrane surrounding most tissues. It has been demonstrated that MT1-MMP, but not other collagenases, can promote invasion into collagen by normal and cancerous cells [17, 41]. Cell migration requires a continuous reorganization of the actin-based cytoskeleton within the cell and coordinated action of adhesion molecules and proteases outside the cell. MT1-MMP localizes at the front of migrating cells called lamellipodium, which facilitates degradation of the ECM barrier, whereas, in some tumour cells, it is found to localize to a specialized structure namely invadopodium [42]. CD44, a multifunctional

glycoprotein involved in cell-cell and cell-ECM interaction is capable to adhere to the ECM via its N-terminal globular domain and has been correlated with MT1-MMP for invasion and migration of cancerous cells. Processing and shedding of CD44 from the cell surface by MT1-MMP enhances cell migration [18]. However, the exact mechanism behind the MT1-MMP/CD44-mediated promotion of cell migration is currently unclear. Recent study has revealed that PKC-mediated phosphorylation of endogenous MT1-MMP could also be an important factor in inducing invasion and migration [43]. Na^+/H^+ exchanger 1 (NHE1), an important regulator of membrane potential is shown to regulate tumour cell invasion and migration partly via regulating expression and localization of MT1-MMP, although the mechanism is still uncertain [44]. MT1-MMP also cleaves and sheds ectodomain of syndecan-1, a transmembrane heparan sulfate proteoglycans expressed on all adherent cells, which stimulates cell migration on collagen matrix [45]. Thus, it seems possible that the ectodomain alone could function as a positive regulator for invasion without influencing MT1-MMP expression, whereas the full-length syndecan-1 would suppress invasion via suppressing MT1-MMP expression [46]. Elucidation of the mechanism could provide important clues for better understanding of how MT1-MMP promotes cell migration. MT1-MMP has also been found to be responsible for cell motility via cleavage of laminin-5 and 10, a major component of basal lamina [47, 48].

4 MT1-MMP in Angiogenesis

Angiogenesis is a complex process involving formation of new blood vessels from the pre-existing one in order to provide oxygen and nutrient supply. Angiogenesis underlies a number of biologic processes including tumor development and metastasis, chronic inflammatory disorders and cardiovascular diseases [49]. Angiogenesis is an invasive process that requires multiple steps like proteolytic processing of the ECM, migration and proliferation of endothelial cells, release of pro-angiogenic factors into the matrix, adherence of the endothelial cells to each other as well as synthesis of new matrix proteins. The role of MT1-MMP in angiogenesis has been reported by several investigators [50–53]. Zhou et al. [54] have demonstrated that MT1-MMP deficient mice exhibit complete absence of neovascularization in response to fibroblast growth factor-2 (FGF-2) suggesting the crucial role of MT1-MMP in angiogenic process. The pro-angiogenic effect of MT1-MMP has been well documented by its ability to process the anti-angiogenic molecule decorin and hence facilitating corneal angiogenesis [55]. Recent studies have also substantiated the role of MT1-MMP in tumour associated angiogenesis, which involves MT1-MMP mediated processing and shedding of soluble factors such as endoglin (a transforming growth factor-β co receptor) and semaphorin 4D (a cell surface protein that regulate axonal growth) [56, 57]. MT1-MMP is required for fibrinolysis, an important aspect for progression of angiogenesis, in plasmin deficient mouse model [15, 58]. Interestingly, MT1-MMP, but neither MMP-2 nor MMP-9, was found to be essential

in neo vessel formation in 3D type 1 collagen matrix [59]. The phenomenon was also supported by another group who demonstrated that anti-MT1-MMP antibody and its endogenous inhibitors, TIMP-2, -3 and -4 blocked angiogenic growth of blood vessels in mouse aortic rings; whereas the disruption of other gelatinases such as MMP-2 and MMP-9 did not have any effect on vascular growth [60]. Elevated level of MT1-MMP has been correlated with increased activation of MMP-2 during sprouting of endothelial tubes from pre-existing microvessels [61].

5 MT1-MMP in Tumour Invasion and Metastasis

MT1-MMP has been demonstrated to be expressed in different tumor cells along with active MMP-2 [6, 62–64]. MT1-MMP expressed on the surface of tumor cells degrades some ECM components such as decorin and lumican, which reinstates tumorigenicity [65]. In fact, MT1-MMP was first identified in membrane fraction of tumour cell surface [6]. MT1-MMP is essential for the remodeling and invasion of collagen matrix of basement membranes of cancer cells [41, 66, 67]. In chick chorioallantoic membrane (CAM), breast cancer cells expressing MT1-MMP promotes pro-invasive and metastatic activities [68]. However, in a similar study performed in the CAM, the down regulation of MT1-MMP did not affect the invasive activity of cancer cells [69]. Involvement of MT1-MMP has been demonstrated to be responsible in human tumor tissues through cleavage of CD44 [70]. CD44 acts as a linker that connects MT1-MMP to the actin cytoskeleton and play a role in guiding MT1-MMP to the invasive front. Elevated level of MT1-MMP is shown to be correlated with blood vessel invasion in biopsies from triple-negative breast cancers [71]. In different tumour invasion models, loss of MT1-MMP activity was poorly compensated by other enzymes [17, 41], suggesting role of MT1-MMP as a potential therapeutic target in prevention of cancer metastasis. The expression of MT1-MMP has been reported to be associated with the malignancy of different types of tumors such as lung [6, 64, 72], gastric [62, 73], breast [63, 74, 75], colon [74, 76], head and neck cancer [77] cervical carcinoma [78] and melanomas [79]. Up regulation of MT1-MMP has been documented to be responsible for epithelial to mesenchymal transition (EMT), a stage for cancer progression from early stage to invasive or metastatic phase [80]. Overexpression of MT1-MMP in human breast cancer cell line enhances tumour growth and metastasis via src kinase mediated up regulation of vascular endothelial growth factor A (VEGF-A) transcription [81, 82]. Several workers have suggested that MT1-MMP is enriched at invadopodia, specialized membrane protruding invasive front that concentrate protease in cancerous cells in contact with ECM and can degrade the matrix [83–85]. Recently, it has been demonstrated that the cleavage of the PGD↓L^{50} sequence of the prodomain of MT1-MMP is necessary for its pro invasive function [86]. Deficiency of MT1-MMP has been reported to have reduced ability to metastasize in mouse lung model [87]. Moreover, stromal fibroblasts isolated from MT1-MMP-deficient tumors did not degrade type I collagen suggesting essential role of MT1-MMP in dissemination of

tumor metastasis [87]. It also came into light in recent report that MT1-MMP prevents collagen induced apoptosis, an internal mechanism for evading cancer progression, in breast cancer cells [88]. Inhibition of internalization or recycling of MT1-MMP, which was found to be mediated via rab7 and vesicle-associated membrane protein 7 (VAMP7), significantly reduced the ability of tumour cells to invade through Matrigel [43]. Moreover, it was found that phosphorylation of MT1-MMP at Thr^{567} in its cytoplasmic tail resulted in down regulation of its expression on the cell surface, which may have implication in reduced cancer cell invasion [43].

6 MT1-MMP in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disorder in which the immune system of our body mistakenly attacks body's own tissues, specifically the synovium, a thin membrane that lines the joints. It is characterized by the overgrowth of synovium, which requires degradation of the ECM and progressive destruction of articular cartilage. MT1-MMP has been reported to be expressed by the cells in the synovium of human RA [89, 90] and cells at the cartilage pannus junction [90]. Involvement of MT1-MMP in cartilage invasion by RA synovial cells by activation of proMMP-2 or by itself has been reported [89–91]. Requirement of MT1-MMP was found to be essential during synovial invasion in human RA [92], suggesting MT1-MMP to be a crucial therapeutic target for RA. Deficiency of MT1-MMP completely blocks type I collagen degradation by RA synoviocytes [93]. In RA synovium, abundant expression of MT1-MMP has been demonstrated, with MT1-MMP being synthesised by synovial fibroblasts, CD68 positive osteoclasts and macrophages [94]. In RA patients increased level of MT1-MMP along with MMP-2 and MMP-13 mRNA was detected in fibroblast-like cells of synovial membrane, implicating their concerted potential in proteolysis mediated destruction of extracellular matrix components and hence in invading adjacent bone and cartilage structures [95]. Interestingly, marked expression of MT1-MMP was also noticed in endothelial cells which might have a consequence in new blood vessel formation in RA [95].

7 MT1-MMP in Ischemia/Reperfusion

Ischemic (deprivation of oxygen supply to the tissue) condition may ensue immediately after stroke, haemorrhagic shock and myocardial infarction, which results in severe tissue damage. However, reperfusion of blood flow to the tissue *per se* may aggravate tissue injury in excess of that produced by ischaemia alone. The cellular complications following reperfusion of previously viable ischaemic tissues is known as ischaemia/reperfusion (I/R) injury. MMPs have been implicated in various physiological dysfunctions following I/R injury [96]. Among the MMPs, MT1-MMP is of special importance. MT1-MMP has been shown previously to be elevated at

transcriptional level during prolonged I/R injury to microvascular endothelial cells [97]. Increased abundance of MT1-MMP in isolated myocytes have been reported in patients with dilated cardiomyopathy (DCM) as well as in response to specific neurohormonal stimulant expressed during the progression of LV dysfunction [98, 99]. Increased abundance of MT1-MMP during post I/R remodeling is believed to be the summation of diminished levels of its inhibitors (TIMP-3 and -4) as well as increased trafficking of the enzyme from the intracellular compartment to the membrane [100]. Involvement of endothelin/PKC axis has been shown responsible for the signaling pathway in mediating increased MT1-MMP activity in I/R injury model providing promising target for attenuation of the injury [101]. On the contrary, the contribution of MT1-MMP in I/R could also be speculated as reconstructive. As in case of a liver I/R injury model, MT1-MMP was found to be elevated at the later stages of injury implicating its possible role in tissue repair process [102]. Differential response in interstitial MT1-MMP activity in case of chronic I/R event contributes to deleterious consequences. In myocardial tissue isolated from pigs, MT1-MMP is increased in post myocardial infarcted tissue that was affected by a second I/R event [103] and is believed to be associated with left ventricular dysfunction.

8 Role of MT1-MMP in Signal Transduction

Accumulating evidence suggests that beside proinvasive effects, MT1-MMP is also responsible for a multitude of signal transduction events. Overexpression of MT1-MMP was documented to be an important phenomenon in the activation of the extracellular signal-regulated protein kinase (ERK) cascade essential for MT1-MMP mediated cell migration [104]. Interestingly, in addition to the catalytic domain, the cytoplasmic domain of the protein was also responsible for the activation process and hence in cell migration [104]. Previous report demonstrates that MT1-MMP works in a positive feedback manner to induce sustained ERK activation, which subsequently stimulates cell migration on collagen matrix (Fig. 2) [105]. The inevitability of the catalytic function of MT1-MMP in mediating its effect was nullified by the fact that in human glioblastoma cell lines MT1-MMP-mediated signaling cascade leads to activation of COX-2 expression that is independent of MT1-MMP's catalytic function [106]. MT1-MMP regulates vascular smooth muscle cell (VSMC)/pericyte function owing to its role in platelet-derived growth factor-B (PDGF-B)/PDGF receptor-β (PDGFRβ) interaction by being directly associated with PDGFRβ [107]. The tumourigenic effect of MT1-MMP is not only limited to its effect in activating proMMP-2 or in matrix degradation. MT1-MMP was reported to activate vascular endothelial growth factor (VEGF), an angiogenic factor through activation of src kinase (Fig. 2) [82]. Although, ERK activation was shown to be dependent upon the cytosolic tail of the enzyme, in this case both cytosolic tail and catalytic domain appeared to be associated with the process [82]. MT1-MMP has been suggested to be involved in pathogenesis of vascular disease related to diabetes. Advanced glycation end products (AGEs) formed in hyperglycemic conditions may contribute to the pathophysiology of vascular

diseases in diabetes. In cultured SMCs, receptor for AGE (RAGE)/MT1-MMP axis plays crucial role in Rac1/NADPH oxidase activation suggesting the axis as a therapeutic target for complications in vascular responses in diabetes [108]. The catalytic active MT1-MMP proteolytically inactivates ADAM9 (Fig. 2) by forming a complex with fibroblast growth factor receptor 2 (FGFR2) and ADAM9 (A Disintegrin And Metalloprotease-9) in osteoblasts and thereby protects FGFR2 from ADAM9-mediated ectodomain shedding on the cell surface [109]. MT1-MMP directly cleaves the Notch ligand Delta-like 1(Dll1) to negatively regulate Notch signalling for maintenance of typical B-cell development in bone marrow (Fig. 2) [110]. Intracellular domain of MT1-MMP in concert with hypoxia inducible factor-1α (HIF-1α) facilitates recruitment and binding of an adaptor protein 3BP2 (c-Abl Src homology 3 domain-binding protein-2) to cell surface facilitating migration of mesenchymal stromal cells (MSCs) implicating transition to hypoxic tumours [111].

9 MT1-MMP in Atherosclerosis

Atherosclerosis or hardening of the arteries in which plaque, made up primarily of cholesterol and fat, builds up inside arteries is the leading cause of various types of cardiovascular diseases. MT1-MMP has been suggested to be associated with progression of plaque formation in atherosclerotic disease. Activation of SMCs and macrophage by proinflammatory molecules such as IL-1α, TNF-α, and ox-LDL elevates MT1-MMP expression in atherosclerotic arteries and thus may influence extracellular matrix remodeling in the context of the disease [112]. Recent *in vivo* study in rabbit demonstrated co-distribution of MT1-MMP and MMP-2 in atherosclerotic lesions, suggesting a possible role for MT1-MMP in destabilization of atherosclerotic plaques [113], which has also been extended in macrophage of human atherosclerotic plaque [114]. Deficiency of MT1-MMP from bone marrow-derived cells decreases the collagen content of mouse atheroma, suggesting a dual role of MT1-MMP in collagenolysis and plaque stabilization [115].

10 MT1-MMP in Bone Resorption

Bone resorption is a catabolic process by which bone is broken down by osteoclasts so it can be replaced by new bone tissue. Excessive bone resorption compared to its synthesis is the major cause of many bone associated diseases, such as osteoporosis. The process consists of multiple steps, including osteoclast differentiation, activation of osteoclast function, degradation of bone matrix proteins, and bone mineral mobilization. Degradation of the bone matrix depends on the activity of proteolytic enzymes, including MT1-MMP. Previous reports have demonstrated that membrane type MT1-MMP was co expressed with proMMP-2 in mouse embryonic osteoblasts

[116], which may participate in initiation of bone resorption by degrading the unmineralized osteoid layer of the bone surface to allow osteoclasts to attach to the mineralized matrix. In rabbit osteoclasts an increase in the level of MT1-MMP has been suggested to play role in the invasive activity of the cells [117]. In vivo study reported localization of MT1-MMP in sealing zone of osteoclasts [118]. The correlation between MT1-MMP expression and bone resorption may not be that simple, as down regulation of MT1-MMP expression by parathyroid hormone activates intracellular signaling pathway in osteoblasts which appears to facilitate bone resorption.

11 Involvement of MT1-MMP in Disorders Associated with Diabetes

MT1-MMP plays a prominent role in severity and progression of diabetes. In a rat model of type 2 diabetes (Zucker diabetic fatty rats), MT1-MMP contributes to progression of the disease as in case of administration of PD166793, broad spectrum inhibitor of MT1-MMP, in diabetic rats prevented both loss of β-cell mass and hyperglycemia in the rat [119]. MT1-MMP also aggravates pathogenesis of type 1 diabetes by modulating the functionality of T cell CD44 receptor, a marker of activated T cells. The pathogenesis of type 1 diabetes begins with the activation of T cells, which then infiltrates into the pancreatic islets and damages the pancreatic β cells. MT1-MMP mediated proteolysis of CD44 receptor facilitates the transendothelial migration of T cells, and thereby, the T cells infiltrate the pancreatic islets causing destruction of β-cell mass [120]. Inhibitor of MT1-MMP has been proved to be effective in lessening the diabetogenic capacity of T cells by stimulating the functional recovery of insulin producing β-cells and the regeneration of the pancreatic islets, which may provide a mechanistic support for clinical trials of the inhibitors of MT1-MMP in type I diabetes patients [120, 121]. Total and active MT1-MMP has been shown to be up regulated in feto-placental endothelium of gestational diabetes mellitus subjects, which may contribute to changes in the feto-placental vasculature [122]. In first-trimester placental samples of type 1 diabetic women, proMT1-MMP has been shown to be up regulated, whereas active MT1-MMP expression was elevated at later stages of first trimester, which appears to have consequences in placental development and in structural alterations in the placenta in pregestational diabetes [123]. Increased expression of MT1-MMP is likely associated with a proteolytically active atmosphere, which may help in remodeling of gingival tissue in animal model of type I diabetes [124]. Reduced expression of MT1-MMP has also been proposed to have major consequences with the severity of diabetes associated complications. Increased deposition and accumulation of ECM proteins due to impaired expression and localization of MT1-MMP on the plasma membrane of glomerular tissue accounts for thickening of the glomerular basement membrane (GBM) of diabetic animals and hence seems to be responsible for disruption of the glomerular filtration integrity [125].

12 MT1-MMP in Lung Development and in Lung Diseases

During fetal lung development, MT1-MMP appears to contribute to the formation of airways and alveoli. In a rabbit model, MT1-MMP was found to be distributed throughout the early stages of developing lung that could ultimately be involved in the formation of larger alveolar surface and branched morphogenesis in developing rabbit lungs [126]. The contribution of MT1-MMP in normal lung development was further supported by its activation in epidermal growth factor receptor (egfr) signaling in mouse model [127]. MT1-MMP$^{-/-}$ mice lungs exhibited reduction in alveolar surface area at early stages and irregular ultrastructural appearance in airways and alveoli [128], which may be a consequence of impaired angiogenesis [129]. Interestingly, analyses of tissue specimen from MMP-2 deficient mice fail to display anomalies in normal lung development similar to those in MT1-MMP deficient mice [129], leading to the proposition that altered lung tissue morphogenesis during normal development could be attributable to the presence of MT1-MMP, but not from the failure to activate proMMP-2. Moreover, MT1-MMP deficient mice display severe defect in airway epithelial regeneration after experimental lung injury with naphthalene [130]. Cigarette smoke derived acrolein has been shown to be responsible for increased expression of MT1-MMP leading to excessive mucin production, a principal characteristic of chronic obstructive pulmonary disease (COPD) [131]. Elevated level of MT1-MMP present in bronchoalveolar lavage fluid (BALF) from bronchial asthma patients has been implicated in human inflammatory lung disease [132]. Exaggerated expression of MT1-MMP in the lung cells has also been reported in case of idiopathic pulmonary fibrosis (IPF), a chronic form of lung disease characterized by fibrosis of the supporting framework [133].

13 Conclusions and Future Perspective

Since its discovery, it has become obvious that MT1-MMP is involved in a multitude of physiological as well as pathological processes. Although, MT1-MMP has been a subject to a variety of clinical therapeutic studies, it is unknown why in some cases MT1-MMP inhibition might interfere with and in others promote progression of disease. Various details of MT1-MMP functions such as transcriptional regulation, intracellular trafficking, inhibition potency of enzyme activity by synthetic inhibitors as well as pericellular targeting remain to be elucidated to fully understand its role in the progression of a variety of disease processes. Our current knowledge suggests an important role for MT1-MMP in cancer metastasis, angiogenesis, atherosclerosis or in RA; however, for clinical strategies in the treatment of different pathophysiological processes, a better understanding of MT1-MMP biology is required. Novel strategies are also required to design to control specific MT1-MMP actions, which may prove useful in the treatment of progression of diseases such as melanoma, atherosclerosis and RA.

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Role of Proteases During Intra-erythrocytic Developmental Cycle of Human Malaria Parasite *Plasmodium falciparum*

Sumit Rathore, Shaifali Jain, MohD Asad, Gaurav Datta, Pawan Malhotra, and Asif Mohmmed

Abstract Malaria remains a major parasitic disease in the tropical and sub-tropical countries mainly due to dramatic increase in parasite lines resistant to commonly used anti-malarials. Characterization of novel metabolic pathways in the parasites and understanding their functional role is a prerequisite to design new anti-malarial strategies. Parasite proteases play key role in growth and differentiation of all the developmental stages across the parasite life cycle and present the most promising targets to develop new drugs against malaria. In *Plasmodium falciparum* genome database a total of 123 proteases are identified; these proteases belong to five different clans: Cysteine, Aspartic, Serine, Metallo-, and Threonine. Some of the most studied parasite proteases are those that are functional in the asexual blood stage cycle. Starting with the processing of key parasite ligand in merozoite, the invasive form of blood stage parasite, degradation of host hemoglobin in food-vacuole, regulation of levels of key metabolic pathways in cytosol and cellular organelles, degradation of misfolded and unused proteins, and rupture of host membrane for egress of daughter merozoites is mediated by these proteases. Here we discuss roles of some of the parasite proteases involved in various steps of the parasite intra-erythrocytic cycle.

Keywords Malaria • *Plasmodium falciparum* • Intra-erythrocytic cycle • Merozoite invasion • Merozoite egress • Hemoglobin degradation • Organelle proteases

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1 Introduction

The phylum Apicomplexa includes various protozoan pathogens causing major parasite diseases in the developing world; the most important of these diseases is malaria which causes about 1 million deaths globally per year [1]. Malaria is caused by five different species of genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*; among them *P. falciparum* is responsible for the most deadly form of malaria infections. *Plasmodium* has a complex life cycle which is completed in three major phases in two host systems. Infection in humans begins with a bite of infected female Anopheles mosquito that injects invasive form of the parasite, sporozoites, which reaches to liver hepatocytes. The sporozoite enter and exits several hepatocytes by ripping through the plasma-membrane before finally infecting one of the host cell. In the infected hepatocytes the parasite resides in a parasitophorous vacuole (PV), undergoes multiple rounds of mitotic nuclear division and organelle division and subsequently large number of merozoites are formed which are released into the blood stream. Merozoites are the blood stage invasive forms that initiate the asexual blood stage cycle. These merozoites invade the host erythrocyte and reside in the parasitophorous vacuole, wherein it develops into a ring stage form which then subsequently grows to develop into trophozoite and then divides many times to develop into schizont, which then ruptures releasing the newly formed merozoites into the blood stream to continue the cycle. During the asexual cycle some of the parasites differentiate into male and female gametocytes which are taken up by the mosquito during blood-meal. Within the mosquito mid-gut, the gametocytes develop into male and female gametes which undergo fertilization; the zygote formed subsequently develops into motile ookinete. The ookinete burrows itself into the mid-gut wall and encyst on the basal lamina. The oocysts undergoes meiosis and divide to form large number of sporozoites that then invade salivary glands from where they can be again injected into human host. The blood stage asexual cycle is responsible for all the clinical symptoms and pathogenicity in humans; therefore the blood stages of the parasites are the target of most of the drug/vaccine development programs.

In light of rapid increase in parasite populations that have multi drug resistance, there is a need to develop new drug targets against the malaria parasite. Recent developments in the fields of genomic, proteomics and metabolomics research have helped to identify new drug targets. Since it is possible to develop specific inhibitors for proteases that can target the defined active sites, the malarial proteases are among the leading potential targets for developing new modes of chemotherapy [2–4]. The detailed genomic studies have identified a total of 123 proteases in *P. falciparum* genome [5, 6]. A number of these proteases are shown to be involved in the mediation of processes within the erythrocytic cycle; these processes include: rupture of host erythrocyte and egress of merozoites; invasion of merozoites into host erythrocytes; degradation of host hemoglobin etc. Functional importance of parasite proteases have been highlighted by detailed studies, which supported their potential as drug targets. Indeed a number of inhibitors have been designed against cysteine and aspartic protease of the malaria parasite with aim to develop as new anti-malarials. These studies identified lead compounds that can block *in vitro* parasite development

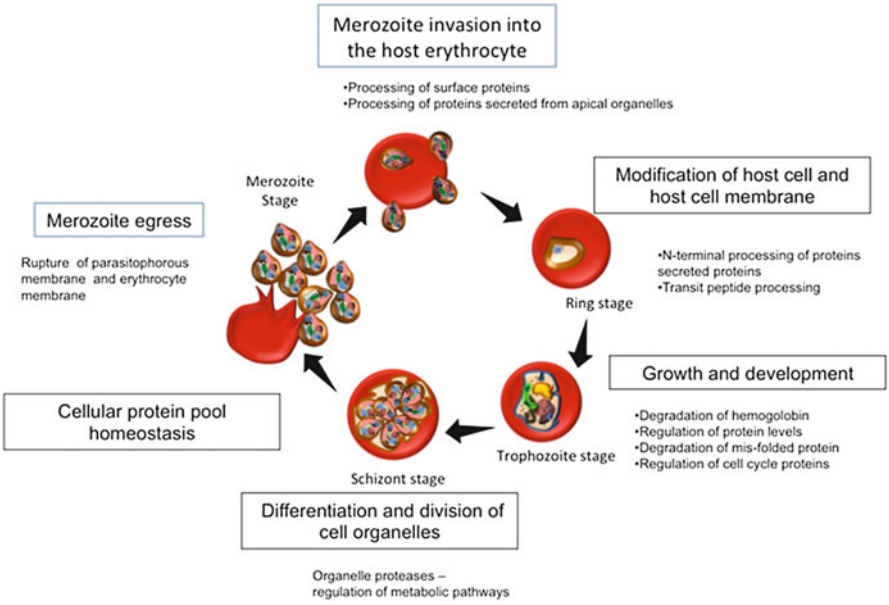

Fig. 1 Asexual erythrocytic cycle of malaria parasite: major developmental stages are depicted and important roles of parasite proteases during these steps are indicated

at nanomolar concentrations and have cured malaria in animal models [7–9]. In this chapter we summarize the role of proteases in the *Plasmodium* asexual life cycle and their potential scope to be developed as drug targets. Two major steps in the asexual life cycle of *Plasmodium* that are majorly dependent on proteases are merozoite invasion and their egress from host erythrocyte; in addition, degradation of host hemoglobin in food vacuole, an essential step in establishment and growth of parasite in the host erythrocyte, is also depends upon various classes of parasite proteases. Proteases in specific cellular organelle and in the cytosol of the parasite also play important role in regulating a number of metabolic pathways and cell cycle (Fig. 1).

2 Role of Proteases During Merozoite Invasion into Host Erythrocyte

Invasion of red blood cells by the malaria merozoite is the first and essential step in the asexual blood stage life cycle of the parasite. The molecular details of invasion of apicomplexan parasite into host cells are only recently becoming understood, and each of these steps is considered as targets of new drug and vaccine development. On coming in contact with the host erythrocyte, *P. falciparum* merozoite re-orients itself such that the apical pole of the parasite points towards and interacts with the host erythrocyte membrane. Two different sets of protein play important role during

interaction of host erythrocyte and merozoites: proteins on the surface of the merozoite that are possibly involved in weak initial attachment with the RBCs; and proteins that are released from the apical secretory organelles of the merozoite, the rhoptries, micronemes, exonemes and dense granules, which are involved in secondary interactions [10]. After merozoite reorientation, the apical organelles release their protein content in sequential manner in response to a calcium-mediated signal [11, 12], most of these released proteins are mobilized to the parasite surface. On the surface, these parasite proteins make high affinity interactions between the parasite and host surfaces. A tight junction forms between the parasite and host, which translocates towards the rear of the parasite via interactions between the microneme proteins' cytoplasmic tails and a cortical parasite actin-myosin system [13, 14]. The role of parasite proteolytic enzymes in these critical steps in the life cycle of *Plasmodium* has been studied extensively. The bulk of the evidence indicates a prime role for serine proteases of the subtilisin and rhomboid families in these steps; these proteases act primarily as maturases and 'sheddases', which are required to process, activate and ultimately remove ligands involved in interactions with the host cell. Processing of some of the major merozoite surface and apical proteins, which play key role in invasion, is mediate by different proteases which points towards the importance of proteases in invasion process. Processing and maturation of some of these important proteins is described here (Fig. 2) (Table 1).

2.1 Merozoite Surface Proteins

The Merozoite Surface Protein-1 (MSP-l), is a GPI anchored protein present in a large protein complex on the surface of *Plasmodium* merozoites [15]. It is suggested to be involved in initial low-affinity binding of the parasite to the host cell, and has been long considered to be a good vaccine candidate. MSP-l is initially expressed as a protein precursor of ~195 kDa, and is subjected to primary processing which is thought to take place whilst the parasites are developing within the host cell rather than during invasion itself. After signal peptide removal and GPI anchor modification, primary processing in *P. falciparum* results in the full length gene product being cleaved into four subunits known as MSP-1_{33}, MSP-l_{30}, MSP-l_{35}, and MSP-l_{42} (in order from the N- to C-terminus of the original gene product and so named based on their molecular weights) [16]. These fragments are bound together non-covalently in a complex, the MSP-l_{42} fragment remains attached on the surface of the merozoite anchoring the complex in the membrane via its GPI anchor. During invasion MSP-l_{42} is proteolytically cleaved into two fragments (called MSP-l_{33} and MSP-l_{19}) in what is known as secondary processing. This processing result in the release of the MSP-l complex from the parasite surface-an event which appears to be important, as only the post-processing stub (MSP-l_{19}) appears to be able to penetrate the moving junction and still be localized to the parasite surface after invasion is complete. The role of proteases in MSP-1 processing as well as shedding has been the subject of intense studies. The first step towards identification of the MSP-1 shedding protease was the observation that this activity is calcium dependent; sensitive

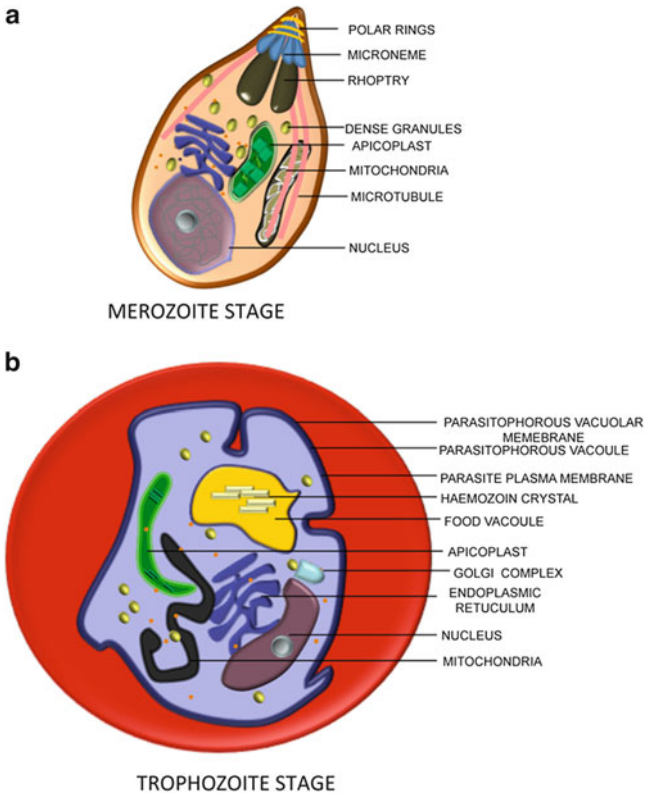

Fig. 2 (**a**) Structure of the Merozoite, the invasive form of asexual erythrocytic cycle of the malaria parasite, showing specialized apical organelles involved in invasion. (**b**) Structure of Trophozoite stage of the parasite in the host erythrocyte; different sub-cellular organelle of the parasite are shown

to the serine protease inhibitors PMSF and DFP, and also that the protease responsible is bound to the parasite plasma membrane when the processing event occurs [17]. Two other important merozoite surface proteins, MSP-6 and MSP-7, also get processed in the parasite. The precursor MSP-6 protein is N-terminal processed to generate MSP-6_{36}. Similarly the precursor MSP-7 protein is N-terminal processed to generate MSP-7_{33}. The MSP-7_{33} gets further cleaved to generate MSP-7_{22} and MSP-7_{11} fragments [18, 19].

2.2 Merozoite Apical Proteins

The *P. falciparum* apical membrane antigen-1 (AMA-l), another long-time vaccine candidate in *Plasmodium* is also shed during invasion and, as for MSPl, anti-AMA-1 antibodies and small peptide based inhibitors that block this processing impede

Table 1 Important parasite proteases involved in processing of merozoite proteins at the time of invasion into host erythrocyte

Protease	Possible important substrates	Localization of substrate in the parasite	Reference
Subtisin-1 (PfSUB1)			
	MSP-1	Merozoite surface	[31]
	MSP-6	Merozoite surface	[31]
	MSP-7	Merozoite surface	[31]
	MSP-9	Merozoite surface	[31]
	SERA4	Parasitophorous vacuole	[31]
	SERA5	Parasitophorous vacuole	[32]
	SERA6	Parasitophorous vacuole	
	RAP1	Apical-rhoptries	[31]
	Rhop3	Apical-rhoptries	[31]
Subtilisin-2 (PfSUB2)			
	MSP-1	Merozoite surface	[34]
	AMA-1	Apical-microneme	[34]
	PTRAMP	Apical-microneme/merozoite surface	[35]
Rhomboid protease-1 (PfROM1)			
	AMA1	Apical-microneme	[55]
Rhomboid protease-4 (PfROM4)			
	MTRAP	Apical-microneme/merozoite surface	[55]
	EBA-175	Apical-microneme	[56]
	BAEBL	Apical-microneme	[55]
	JESEBL	Apical-microneme	[55]
	MAEBL	Apical-rhoptries	[55]
	Rh1	Apical-rhoptries	[55]
	Rh2a	Apical-rhoptries	[55]
	Rh2b	Apical-rhoptries	[55]
	Rh4	Apical-rhoptries	[55]

merozoite invasion [20, 21]. While analyzing the activity responsible for PfAMAl shedding, Howell et al. discovered that PfAMAl is shed by a protease with the same characteristics and inhibition profile as that responsible for MSP-l shedding [22, 23]. They concluded that the same protease, named Merozoite Surface Sheddase (MESH,) (which was later defined as Subtilisins) is responsible for the shedding of the two proteins (Fig. 3).

2.3 Role of Subtilisin-Like Serine Proteases During Invasion

The vital role of MESH during invasion of RBCs by the merozoites led investigators to search for candidate proteases in *Plasmodium*. The subtilisin-like family of proteases emerged as primary candidates owing to their calcium dependent serine protease activity and late stage expression pattern; characteristics' similar to that of a putative MESH. Three *P. falciparum* genes encoding products belonging to the

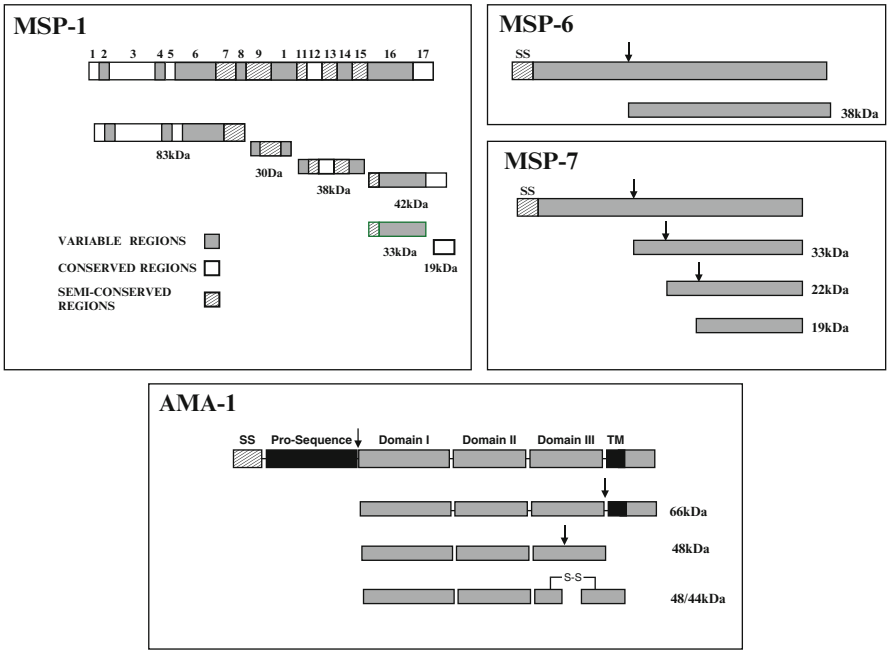

Fig. 3 Primary Structure and processing of *P. falciparum* merozoite surface/apical proteins: MSP-1, MSP-6, MSP-7 and AMA-1. The primary precursor MSP-1 protein contains a number of variable, conserved and semi-conserved regions. Primary processing of this protein generate fragments of different sizes labelled as MSP-1_{83}, MSP1-$_{30}$, MSP1-$_{38}$ and MSP1-$_{42}$; during invasion the MSP1-$_{42}$ gets further cleaved into MSP1-$_{33}$ and MSP1-$_{19}$ [16]. The precursor MSP-6 protein is N-terminal processed to generate MSP-6_{36}. Similarly the precursor MSP-7 protein is N-terminal processed to generate MSP-7_{33}. The MSP-7_{33} gets further cleaved to generate MSP-7_{22} and MSP-711 fragments [18, 19]. AMA-1 is expressed as 83 kDa precursor protein consisting of N-terminal pro-sequence and a C-terminal trans-membrane region. In the micronemes the pro-sequence is cleaved off leaving 66 kDa protein containing three domains (*I*, *II* and *III*) attached to the membrane and is released on the merozoite surface. The 66 kDa is shed by juxtamembrane cleavage releasing 48 kDa fragment; further processing of this 48 kDa within the Domain *III* generate 44 kDa fragment which remains attached to the small polypeptide comprising the remainder of domain *III* via a intra-molecular disulfide bond [22, 23]

superfamily of subtilisin-like serine proteases, or subtilases, have been identified. Two of these genes, *pfsub-1* and *pfsub-2*, were discovered and their gene products partially characterized some time ago [24, 25], whereas the presence of a third gene, *pfsub-3*, was revealed only by the *P. falciparum* genome project [26]. Both PfSUB-1 and PfSUB-2 are expressed in asexual blood stages and the mature enzymes accumulate in the apical regions of the merozoite. PfSUB1 is localized in special apical secretory organelles, the exonemes, and gets released in response to a calcium dependent signal into the parasitophorous vacuole just prior to schizont rupture and merozoite release [27, 28]. Selective inhibitors of PfSUB-1 do not inhibit shedding of MSP-1 or AMA-1, formally ruling out any involvement of PfSUB-1 in this

process [29]. However, this inhibits egress of blood-stage *P. falciparum*, suggesting that PfSUB-1 is essential for parasite growth. The major role of PfSUB1 is processing of another protease PfSERA5 which is essential for parasite egress (as discussed later in this chapter). Nevertheless, it was later shown that PfSUB1 is required for pre-processing of MSP-1 along with MSP-6 and -7 prior to schizont rupture [30]. Global proteomic studies also identified several of merozoite surface and apical proteins [31] Overall, these and subsequent studies showed that the PfSUB1 thus plays an important role not in the actual invasion process but in priming the merozoites for invasion prior to their release from the schizont [29, 32, 33].

Another plausible candidate for a MESH, that is PfSUB2; a type I integral membrane protein, was identified by two research groups simultaneously [24, 25]. PfSUB2 represents a different sub class of eukaryotic pro-protein convertases as its deduced active site sequence resembles more with the bacterial subtilisins. Molecular modeling studies of PfSUB2 catalytic domain co-related with its proposed substrate specificity [24]. Further, PfSUB2 localization to the dense granules in merozoites made it an ideal candidate protein to function as MESH [23, 24]. Later it was shown that PfSUB2 localizes to the micronemes and is released just after schizont rupture to relocate to merozoite plasma membrane. In the same study it was shown that PfSUB2 specific peptide based inhibitor derived from its prodomain can block MSP-1 and AMA-1 shedding [31]. Another study has shown that apart from MSP1and AMA-1, the PfSUB2 also mediate shedding of another invasion related protein, PTRAMP [34, 35]. Like PfSUB-1, PfSUB2 appears essential for blood-stages of the parasite as attempts to disrupt the *sub2* gene in the rodent malaria *P. berghei* have been unsuccessful [36]. Recently Alam et al. have characterized PfSUB3 from *Plasmodium falciparum* [37].

2.4 Other Invasion Related Proteases: SERA-5, ABRA and Rhomboids

Another important protease family that plays important role in parasite invasion is the Serine repeat antigen (SERA) family. The human malarial parasite *Plasmodium falciparum* possesses nine SERA proteins, which belongs to cysteine protease family. Of these nine SERA proteins, six contains serine at active sites (serine-type) (SERA1 to SERA5 and SERA9) and three have cysteine at the active sites (cysteine-type) (SERA6 to SERA8) SERAs. Miller et al. tried knocking out eight of the nine SERAs located as a cluster on chromosome 2, the peripheral genes SERA-2, -3, -7 and -8 were dispensable the central genes, SERA-4, -5 and -6 remained refractory to deletion [38]. Joanne E. McCoubrie et al. then later tried knocking out four "serine type" SERA proteins; SERA1, SERA4, and SERA9 knockout lines were generated successfully, while SERA5, the most strongly expressed member of the SERA family, and SERA6 remained refractory to genetic deletion [39]. Serine repeat antigen-5 (SERA-5), also referred to simply as SERA, was initially identified as an abundant component of the PV that was shed in a soluble form at merozoite release.

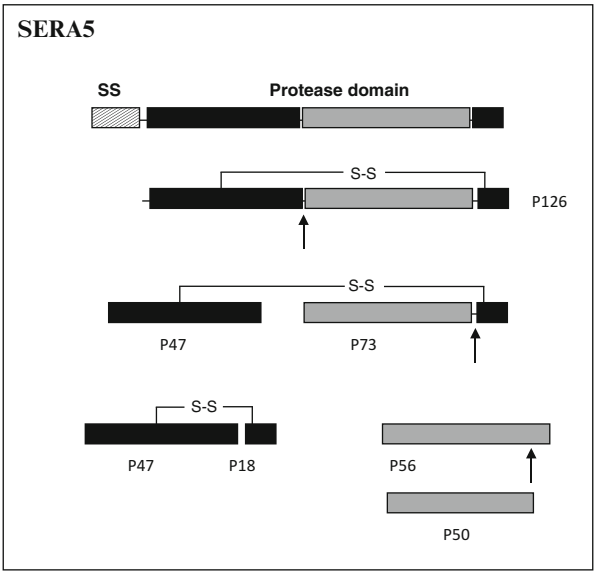

Fig. 4 Primary Structure and processing of SERA5 during egress of *P. falciparum* merozoites from host erythrocytes. The precursor SERA5 (P126) is localized in the parasitophorous vacuole. Cleavage by PfSUB1 at two sites releases P56 which contains a central protease domain. The other two terminal fragments generated (P47 and P18), remain attached with each other due to disulfide-bond; this complex gets attached to the merozoite surface. The P56 fragment plays a proteolytic role during egress; later the P56 fragment is truncated by an unknown cysteine protease to modulate its function, perhaps by inactivation [11]

This and subsequent work [11] indicated that SERA5 was subjected to complex proteolytic processing, and that antibodies against it could interfere with merozoite release and erythrocyte invasion [40]. The central region of the molecule shared homology with the papain-like cysteine protease family, with the significant difference that the residue at the position of the active-site cysteine was replaced in SERA5 with a serine [41]. It is suggested that SERA5 can act as a protease despite its unusual active-site serine; recent studies showed that recombinant SERA-5 possesses autolytic activity, as well as chymotrypsin-like protease activity in trans against peptide substrates (Fig. 4) (Table 2) [42].

The probable role of SERA5 in invasion process is pointed out by processed proteolytic fragments derived from the N- and C-terminal regions of SERA-5, which associate with the merozoite surface [43, 44]. The significance of this is unclear, but it has been suggested that SERA-5 may play a role predominantly in merozoite release rather than invasion [42]. Later studies have linked the processing PfSERA5 in the parasitophorous vacuole and rupture of PVM during egress [33, 45]. Indeed, inhibition of processing of SERA5 shown to block the rupture of schizonts and release of merozoites [28]. Another member of this family, SERA-6 is also localized in the parasitophorous vacuole also gets processed by PfSUB1 to become active protein; SERA6 is also associated with egress and is essential for parasite survival [46].

Table 2 Important parasite proteases that play critical role in different steps of the asexual erythrocytic life cycle

Class/name	Localization	Reference
Invasion		
Subtilases		
SUB2	Microneme	[35, 36]
SUB3	*	[37]
Rhomboids		
ROM1	Microneme	[55]
ROM4	Merozoite surface	[55]
SERA5	Parasitophorus vacuole	[38]
ABRA	Merozoite surface/PV	[50]
Falcipain1	Dense granules	[60]
Egress		
SUB1	Exoneme	[29]
DPAP3	* (PV)	[32]
Plasmepsin II	Food vacuole/PV	[64]
Falcipain 2	Food vacuole/PV	[66]
Hemoglobin degradation		
Plasmepsins		
Plasmepsin I	Food vacuole	[71]
Plasmepsin II	Food vacuole/PV	[64]
Plasmepsin IV	Food vacuole	[71]
Histo aspartic protease	Food vacuole	[71]
Falcipains		
Falcipain 2	Food vacuole/PV	[66]
Falcipain 2′	Food vacuole	[74]
Falcipain 3	Food vacuole	[74]
Falcilysin	Food vacuole/apicoplast/mitochondrion	[78]
DPAP1	Food vacuole	[156]
Aminopeptidases		
M1AAP	Food vacuole/PV	[103]
M17AAP	Cytosol	[101]
M18AAP	Cytosol	[105]
Organelle proteases		
Mitochondrial proteases		
ClpQ	Mitochondria	[116]
Falcilysin	Food vacuole/apicoplast/mitochondrion	[78]
Apicoplast proteases		
ClpP	Apicoplast	[30]
Falcilysin	Food vacuole/apicoplast/mitochondrion	[78]
Stromal processing peptidase	Apicoplast	[128]
ER		
Plasmepsin V	ER	[155]
Signal peptide peptidase	ER	[152]
Other cellular proteases		
Proteasome	Cytoplasm	
UCHL3	Cytoplasm	[140]
UCH54	Cytoplasm	[140]

* The localization of that protease in the parasite is not known

Another *P. falciparum* merozoite peripheral surface protein proposed to mediate serine protease activity is known as acid basic repeat antigen, or ABRA. *P. falciparum* ABRA is a protein of about 100 kDa in size that accumulates during schizont maturation in the PV in a soluble form, but is also bound to the merozoite surface. Its name is derived from the presence within its sequence of two regions of highly charged tandem peptide repeats. The primary structure does not contain recognizable sequence motifs characteristic of major serine protease clans. The first suspicions that ABRA might be a protease came from the observation that the purified parasite protein consistently exhibited chymostatin-sensitive protease activity [47]. Recombinant ABRA produced in bacteria also appeared to possess proteolytic activity and the catalytic region was mapped to the N terminal domain of the protein that contains a serine residue, Ser317, previously proposed on the basis of sequence comparisons to be the active-site serine [47–49]. Possible role of ABRA has been suggested in erythrocyte binding during invasion [50]; however, importance of the predicted active-site Ser317 is not very clear. Clear orthologues of ABRA have been identified in *P. vivax* and two simian malarias [51] but an alignment of these sequences with that of ABRA shows that Ser317 is not conserved across species, being replaced by Glu in all the other sequences. It was found that disruption of the gene encoding MSP-3 resulted in the expression of truncated protein, which prevented trafficking of both MSP-3 and ABRA to the parasitophorous vacuole and merozoite surface [52]; however the resulting transgenic parasites lacking surface forms of both MSP-3 and ABRA were still capable of *in vitro* growth, which suggest that ABRA may not be playing a direct role in merozoite invasion.

As mentioned above, shedding of at least some *Toxoplasma* tachyzoite microneme proteins is mediated by a protease activity with the characteristics of rhomboids, which cleave within the TMD of integral membrane proteins [53]. Genes encoding rhomboid-like proteins are evident in the annotated *P. falciparum* genome [5, 6]. Earlier studies indicated that an activity of this nature may be present at the merozoite surface [54]. Later detailed studies using mammalian expression system showed that *Plasmodium falciparum* rhomboid protease PfROM-1 may be involved in cleavage of PfAMA-1 whereas PfROM-4 may be also involved in cleaving diverse adhesins including TRAP, CTRP, MTRAP, EBA-175, BAEBL, JESEBL, MAEBL, Rh1, Rh2a, Rh2b, and Rh4 [55, 56]; it was also shown that this cleavage relied on the adhesin transmembrane domains. However, later ROM-1 was shown to play role in sporozoite stage invasion and establishment of parasite into host hepatocyte [57, 58].

Another important parasite protease for which experimentally demonstrated link with invasion was shown is the Falcipain-1, an important cysteine protease of the parasite. Falcipain 1 was the first identified member of a small family of papain-like *Plasmodium* cysteine proteases; it was originally characterized as being primarily involved in haemoglobin catabolism during intra-erythrocytic growth [59]. However, studies with a radiolabelled cysteine protease chemical probe demonstrated, contrary to what would be predicted of a haemoglobinase, the Falcipain-1 expression peaks in merozoite and ring (the newly invaded parasite) stages of the erythrocytic cycle [60]. Indeed the enzyme was also localized at the apical end of the merozoite [60]. Treatment of cultures with falcipain 1 inhibitors derived from a positional

scanning peptidyl epoxide library had no effect on intracellular growth of the parasite but appeared to very effectively prevent invasion by released merozoites, leading to the proposal that this protease has an important role in invasion. Some doubt was cast on this interpretation, however, by the recent demonstration that disruption of the *P. falciparum falcipain-1* gene has no detectable effect on replication of asexual blood-stage parasites [61]. Although it is possible that up-regulation of other proteases may have compensated for the absence of Falcipain-1 in the knockout parasites, this work however proves that Falcipain-1 is not absolutely essential for replication of the asexual blood-stage parasite. As a result—and although its function remains obscure—the protease is unlikely to be considered a good target for anti-malarial drug development.

3 Role of Proteases During Rupture of Host Erythrocyte and Merozoite Egress

Rupture of host erythrocyte membrane and egress of merozoite into host milieu is a complicated process involving many steps; role of several and different classes of parasite proteases is suggested among these steps. In the early 1980s, the role of proteases in the mechanism of egress was pointed by Banyal et al. [62]. A number of serine and cysteine inhibitors have been studied for their effect on the egress of *P. knowlesi* merozoites. It was observed that mature schizonts accumulated upon treatment with a mixture of leupeptin, Chymostatin, antipain (a serine and cysteine protease inhibitor) and pepstatin (an aspartic protease inhibitor). Detailed studies showed that the process of egress is a two-step process, involving primary rupture of the parasitophorous vacuole membrane followed by a secondary rupture of the erythrocyte plasma membrane [63]. Using specific inhibitors and transgenic lines expressing GFP in different compartment of the infected erythrocyte, it was shown that the each step is mediated by distinct proteases; the primary vacuolar lysis step can be inhibited by cysteine protease inhibitors E-64 whereas the leupeptin and antipain can inhibit secondary erythrocyte rupture step [63]. Overall the egress may involve several proteases and their sequential processing by other enzymes. Proteases that have been implicated in parasite egress are: (1) aspartic proteases e.g. Plasmepsins and histo-aspartic proteases [64] (2) cysteine proteases e.g. falcipains [65, 66] (3) dipeptidyl peptidase 3 (PfDPAP3) [32] (4) Serine Repeat Antigens (SERAs)[38, 67]; and (5) serine protease subtilase 1 (PfSUB1) in the subtilisin S8 family [29].

The *Plasmodium* aspartic and cysteine proteases (plasmepsins and falcipains respectively) have been shown to function primarily as hemoglobinases in the parasite food vacuole as discussed later in this chapter. However, some evidence points towards their dual functionality as some members being also involved in the process of parasite egress from the host erythrocytes. The ability of Plasmepsin II to digest the host RBC cytoskeletal proteins like spectrin and actin and its localization in the host RBC cytosol outside the parasite provided the first indication towards this dual functionality and the possible involvement of food vacuole protease in the process of egress [64]. Similarly, Falcipain-2 was also demonstrated to be able to digest ankyrin

and protein 4.1 at neutral pH [68, 69]. Further Dhawan et al. were able to inhibit the activity of recombinant Falcipain-2 using a peptide based on the cleavage site in ankyrin [65]. Transient silencing of Falcipain-2 also caused inhibition or merozoite egress in *P. falciparum* [70]. Subsequent gene disruption studies have shown that neither Plasmepsin II nor falcipain 2 is essential in asexual blood stages, and the knockout lines showed no defect in parasite egress from the host RBC [71–74]. However, the loss of falcipain2 was accompanied by an increased transcription of another similar gene falcipain 2′ [72]. The role of these proteases in egress cannot be completely ruled out but it is clear that there is some degree of redundancy involved which requires further attention. DPAP3 is a cathepsin-like cysteine protease identified as an important protease required for egress in an inhibitor based screening [32]. In the same study it was found that the inhibition of DPAP3 caused loss of PfSUB1indicating that it may be playing a role in PfSUB1 folding and activation and the inactive forms of PfSUB1 might be rapidly degraded. As described above, PfSUB1 is released from the exonemes just prior to schizont rupture in response to a calcium dependent signal into the PV where it carries out cleavage of SERA-5 along with other SERA proteins [29] which subsequently play key role in egress.

In addition to the parasite proteases, a host calcium-dependent protease, Calpain-1, is also required for efficient parasite egress of *Plasmodium* and *Toxoplasma* [75]. A cysteine protease inhibitor (DCG04) does not affect the parasite growth but prevents the release of parasite from the host cell. Selective extraction of treated cells identified host Calpain-1 as the target of this inhibitor. Calpain-1 is shown to be present in the cytoplasm of the infected host cell until the schizont stage of parasite growth, subsequently it shift to the membrane, indicating calcium binding and activation. Calpain-1 removal from erythrocytes prevented parasite egress and led to the growth arrest in the schizont stage, whereas reconstitution with recombinant calpain-1 could restore normal growth development.

4 Hemoglobin Degradation: Food Vacuole Proteases

Plasmodium parasite possesses a limited capacity for de novo synthesis of amino acids; the cellular amino acid pool in these parasites is thus derived from host cell hemoglobin after its degradation in a specialized form of lysosome called the ‘food vacuole.’ Apart from being a nutrient source degradation of hemoglobin is also important to maintain the osmotic integrity of the infected red blood cell. The food vacuole is an acidic compartment with pH around 5.2. Hemoglobin degradation is carried out by several vacuole-located proteases in a semi-ordered fashion. The process starts with an attack on the native hemoglobin. Enzymes capable of such attack include the aspartic proteases plasmepsin-I and plasmepsin-II, which cleave the alpha chain of hemoglobin, breaking the structure and exposing several other sites making it prone to other proteases’ attack. Further degradation process is carried out by the aspartic protease plasmepsin 4 (a histo-aspartic protease) and three falcipain proteins (falcipain 2, falcipain-2′ and falcipain-3), resulting in peptides that are larger than 20 amino acids in length. It has been suggested that falcipain 2 and 3 are

also capable of attacking the native hemoglobin so may participate at the very first step [61, 70, 72, 76, 77]. These peptides in turn are degraded by other peptidases that breaks these peptides to smaller ones, around 5–8 amino acid in length. One of the candidate protease for this step is falcilysin, a zinc metalloprotease localized to multiple parasite compartments and proposed to play diverse function [78]. The final step within the food vacuole is catalyzed by dipeptidyl aminopeptidase 1, an enzyme that produces dipeptides.

4.1 Plasmepsins

The *P. falciparum* genome harbours ten aspartic protease genes (PM I, II, and IV–X and HAP) [71, 73, 79, 80] Out of these, three Plasmepsin (PM VI, VII and VIII) are not expressed in asexual blood stages. Rest all of the Plasmepsins are expressed in asexual stages at different locations. PM I and II are localized in the food vacuole and are considered to be the major players required at the very first step in the process of heamoglobin degradation [73, 81] Plasmepsin I and II carries out the first attack on the hemoglobin alpha chain opening up the structure for further protease cleavage It can then acted upon by other proteases including another plasmepsin, PM IV and Histo-aspartic protease (HAP) [73]. As in case of many of the parasite protease, Plasmepsins I and II are synthesized as pro-enzymes. Removal of the pro-domain is required to release the mature enzyme. Activation can be blocked with two tripeptide aldehyde compounds of low specificity, but the identity of the pro-plasmepsin processing enzyme has not been established yet [79]. The pro-plasmepsin convertase has been suggested as a promising target for new antimalarial drugs, since its blockage would inhibit the formation of all four food vacuole plasmepsins [82].

Malarial parasites, *in vitro* and *in vivo*, can be killed by specific inhibitors of Plasmepsins, indicating that these proteases are viable as drug targets. Analysis of substrate preferences and active site mutations has provided insight into the binding specificities of these different plasmepsins. Design of compounds able to inhibit several plasmepsins could be favorable, not only for efficient killing of the parasites, but also to impede the development of parasite resistance. HIV-1 protease inhibitors has provided a large pool of compounds, successfully utilized in the search for Plasmepsin inhibitors [83–86]. Some of the HIV-1 protease inhibitors currently on the market have demonstrated activity against Plasmepsin II as well as activity in *P. falciparum* infected erythrocytes [87, 88]. In addition these HIV-1 protease inhibitors have shown anti-parasitic activity in a murine malaria model [89].

4.2 Falcipains

There are three falcipains present in food vacuole; Falcipain-2 Falcipain-2′ and Falcipain-3. These cysteine proteases play a major role in degradation of

hemoglobin and thus are most important protease present in food vacuole. Possible role of Falcipain-1 in merozoite invasion and gene deletion studies are discussed earlier in the chapter [60, 61]. FP2 and FP3 are the major hemoglobinases expressed in trophozoite stage, localize to the food vacuole, and degrade hemoglobin [66, 90, 91]. FP2′ is biochemically very similar to FP2, and share high sequence homology with FP2 [76]. Gene knockout and transient silencing analyses have revealed that Falcipain-2 is the major hemoglobinase as its disruption lead to low degradation rate of hemoglobin [70, 72]. Transient silencing of Falicpain-2 homologue, berghepain-2, in mouse malaria model caused inhibition of parasite growth [70, 92]. It is recently shown that FP-2 exists as a component of large protein complex consisting of several other proteases and heme-detoxification protein (HDP), and it is suggested that all these components work in a cooperated manner [77]. Falicpain-3 could not be disrupted which points towards essential role of FP3 in parasite [74]. Being most important of all Falicpains, FP-2 remained as first choice of all against which inhibitors were designed. Several studies are been done to develop lead compounds against FP-2. Different compounds ranging from peptide fluromethyl ketones, peptide vinyl sulfones, peptide aldehydes and a-ketoamides lot of chemical scaffolds have been used to develop inhibitors against FP-2 [93, 94]. In addition, A number of groups have been involved in developing falcipain-2 inhibitors using peptidomimetic approaches [95–98].

4.3 Aminopeptdiase

P. falciparum genome encodes nine exo-aminopeptidases, four of these enzymes are annotated as methionine aminopeptidases function in the catalytic removal of N-terminal initiator methionine during protein synthesis. The remaining five aminopeptidases are potential candidate enzymes for the release of free amino acids from hemoglobin-derived peptides [99]. The intra-erythrocytic stages of the human malaria parasite *P. falciparum* express two neutral metallo-aminopeptidases that are believed to be involved in the terminal stages of host hemoglobin digestion, an M1 alanyl aminopeptidase (PfM1AAP) and an M17 leucine aminopeptidase (PfM17LAP) [100, 101]. The M1 aminopeptidase harbors a trans-membrane domain and so thought to be a membrane protein. However, it was shown to be processed and thus being a soluble protein localized to parasite cytosol and around the food vacuole [102, 103]. Dalal and Kemba later showed using YFP-tagged transgenic line that PfM1AAP is localized to food vacuole and nucleus and not in cytosol [104]. In the same study it was also shown that PfM1AAP is essential as it cannot be knocked out in the parasite along with two other APs; PfM17LAP and aminopeptidase P (PfAPP). PfM18AAP, with highest expression levels in rings, is another member of aminopeptidase family. Functionally active recombinant enzyme, rPfM18AAP, and native enzyme in cytosolic extracts of malaria parasites are 560-kDa octomers that exhibit optimal activity at neutral pH and require the presence of metal ions to maintain enzymatic activity and stability [105]. As in case

of human aspartyl aminopeptidase, the exopeptidase activity of PfM18AAP is exclusive to N-terminal acidic amino acids, glutamate and aspartate, making this enzyme of particular interest and suggesting that it may function alongside the malaria cytosolic neutral aminopeptidases in the release of amino acids from host hemoglobin-derived peptides. Whereas immune-cytochemical studies using transgenic *P. falciparum* parasites show that PfM18AAP is expressed in the cytosol, immunoblotting experiments revealed that the enzyme is also trafficked out of the parasite into the surrounding parasitophorous vacuole. Antisense-mediated knockdown of PfM18AAP results in a lethal phenotype as a result of significant intracellular damage and validates this enzyme as a target at which novel antimalarial drugs could be directed. The importance of parasite aminopeptidase in hemoglobin degradation has made these proteases as potent drug targets against Parasite. A number of structural and bioinformatic studies have been carried out to develop new antimalarial targeting aminopeptidases of *P. falciparum* [106–110].

5 Organelle Proteases and Cell Cycle Regulation

The malaria parasite *Plasmodium* possesses two essential organelles, which have prokaryotic origin, the mitochondrion and the relict plastid apicoplast. Both the organelles are essential for parasite survival and plays essential role in maintenance of parasite cell cycle. The metabolic pathways in the mitochondrion and the apicoplast may represent suitable drug targets in the parasite. Selected antibiotics such as doxycycline and clindamycin which target some of these prokaryotic metabolic pathways have already been shown to possess antiparasitic efficacies and are used in malaria treatments [111–115].

5.1 Mitochondrial Proteases

Plasmodium harbours a single mitochondrion during its asexual cycle which divides just before cytokinesis and is distributed equally as single organelle per progeny. Mitochondrion in *Plasmodium* is a validated drug target as the known antimalarial drug atovaquone acts on the respiratory chain complex III in mitochondrion. The only known proteases to be localized in *P. falciparum* mitochondrion are ClpQ [also called HslV (Heat shock loci V)] [116, 117]. In addition, Falcilysin is shown to have multiple localization in the parasite and only partially localized to mitochondrion while it is majorly involved in haemoglobin degradation in food vacuole and also in transit peptide degradation in the apicoplast [80]. The ClpQ protease is a mitochondrial resident protease machinery having ClpY [also called HslU (Heat shock loci U)] as the ATPase partner. ClpQY is the prokaryotic predecessor of the eukaryotic proteasomal machinery; in the ClpQY machinery the ClpY is the ATPase partner forming a hexameric head over the two hexameric core assemblies of ClpQ protease

either on one or both sides [118]. The ClpQY machinery seems to play an essential role in the growth and survival of the parasite at least during the asexual blood stage as the disruption of the machinery by blocking ClpQ and ClpY interaction leads to parasite death with the death phenotype resembling apoptosis in eukaryotic cells [119]. The ClpQY machinery is essential in regulation of replication of mitochondrial genome in *Trypanosoma brucei* [120] knockdown of ClpQY results in over replication of minicircle DNA and abnormal segregation of kinetoplast leading to formation of large kDNA networks which ultimately blocks cell division. Further, the absence of a homolog in the human host leads to the conclusion that ClpQY protease machinery could be a promising drug target in all apicomplexan parasites. Indeed functional importance of ClpQY in *P. falciparum* is clearly shown. Disruption of ClpQY machinery in *P. falciparum* by using small peptide based inhibitors caused dysfunctioning of mitochondria and inhibited parasite growth [119] further, this initiated a cascade of protease and nuclease activation that caused apoptosis like cell death of the treated parasites [119]. Further, trans-expression of mutant inactive ClpQ protein caused dominant negative effect in the parasite which disrupted mitochondria development and caused parasite death [121]; these studies thus support the essentiality of the ClpQY protease machinery for parasite survival and candidature of ClpQ as a promising target for developing new anti-malarial.

5.2 Apicoplast Proteases

The discovery of the apicoplast in the *Plasmodium spp*. in 1996 instantly made it a key target for the development of new therapies against these pathogens owing to its prokaryotic origin and hence availability of various organelle pathways absent from the human host that could be targeted. The apicoplast is a reduced cyanobacterial plastid in the parasite and was acquired by the apicomplexan protozoans by secondary endosymbiosis. It plays an important role in biosynthesis of haem, isopentenyl diphophate and fatty acids [122], thus the apicoplast is considered to be crucial for parasite survival. Antibacterial agents such as ciprofloxacin, rifampicin and thiostrepton that target DNA replication, transcription and translation of the apicoplast, respectively, have been also shown to kill the parasite [123–126]. It is recently showed that the critical and essential function of the apicoplast is in isoprenoid precursors synthesis, and it possible to generate apicoplast minus parasites *in vitro* by chemically rescue using isoprenoid precursors supplemented media [127].

Apicoplast is a four membrane bound structure having a 35 kb genome; however about 95 % of its proteins are nuclear encoded which are imported via a complex protein translocation pathway which is yet to be fully elucidated. Since, majority of the proteins of the organelle depend on this pathway to be correctly delivered to their respective site of action to carry out their required function it is understandable that any perturbation in this pathway will lead to chaotic situation inside the apicoplast and hence may be lethal for the parasite. The protein import to the apicoplast makes use of a bipartite N-terminal extension of the protein; the first part of this sequence

targets the protein into the secretory pathway while the second part called the transit peptide (TP) region takes the protein through the four surrounding membranes of the organelle. Two proteases have been proposed to take part in this process; the stromal processing peptidase (SPP) is considered to take part in the import process directly by being responsible for cleavage of the transit peptide to yield the mature protein [128] while the second protease falcilysin is indirectly linked with the process and is proposed to be the enzyme responsible for degradation of transit peptide [80].

Another protease localized to the apicoplast is ClpP, a serine type protease functioning in conjunction with an ATPase to form the complete protease machinery as in case of ClpQY. The parasite genome codes for four Clp ATPases termed as ClpB1, ClpB2, ClpC and ClpM and also an inactive version of the ClpP protease termed ClpR [129]. Three of these ATPases, ClpB1, ClpC and ClpM are localized to the apicoplast as did ClpR while ClpB2 localizes to the parasitophorus vacuole [129]. The ClpP protease is localized to the apicoplast and is proteolytically active; a specific inhibitor of ClpP can block apicoplast development caused significant inhibition of parasite growth *in-vitro* [130]. ClpP is thus a promising drug target and its inhibitor can be developed as lead anti-malarials.

6 Other Cellular Proteases

A number of cellular pathways employ proteases that do not directly distinguish their substrates but instead utilize a post-translational modification of the protein as the recognition signal. This includes complex multi-meric ATP dependent protease system, Proteasome.

6.1 Proteasome

The 20S proteasome is a multimeric self-compartmentalising protease machinery essential for the survival of every eukaryotic cell. The machinery consists of two central rings formed by the β subunits capped on both sides by α subunit rings. The diversity of the individual subunits varies among different organisms, ranging from a single subtype for each in archae-bacteria to seven subtypes of each in human. Apart from the basic proteolytic core complex, proteasome also has the 19S regulatory subunit which helps in substrate recognition and unfolding of the substrate driving it through the central proteolytic chamber. The functions of the proteasome range from simply degradation of misfolded proteins destined so by polyubiquitination, to regulation of the cell cycle by maintaining the levels of respective proteins such as cyclins and various transcription factors.

Protista, particularly the pathogenic protists are by far the only known eukaryotes to possess both 26S proteasome in addition to its prokaryotic predecessor ClpQY as described earlier [131]. In-silico studies have identified all the 14

subunits of 20S proteasome along with the subunits of the 19S regulatory particle [5, 132, 133]. However, given the degree of conservation and essentiality of the ubiquitin proteasome pathway it is surprising that despite the sequencing of several parasite genomes so little is known about its specific functional role in any of the parasites. Increasing evidence suggest essentiality of the proteasome machinery for the malaria parasites and hence it being a plausible drug target [134]. The irreversible inhibitor of the proteasome, lactacystin, could stall the growth of *P. bergei* parasite *in-vitro* as well as *in-vivo*. Also, the inhibitory effect of lactacystin on *P. falciparum* parasite was found to be cell cycle specific, the drug being able to kill parasite only if applied prior to initiation of DNA replication and not afterwards. A range of proteasome inhibitors have thus far been tested for their efficacy against the malarial parasite. These inhibitors including, lactacystin, salinosporamide A, MG132, epoxomicin, Thiostrepton and bortezomib were reported to inhibit parasite growth *in vitro* at low nanomolar concentrations [133–137].

6.2 Ubiquitination and Deubiquitinations

Ubiquitination is by far the best characterised post translational modification targeting the proteins for degradation, i.e., by the Proteasome. However, evidence that it also plays a key role in regulation of several other cellular pathways is mounting, wherein it serves as any other protein modification (such as phosphorylation) required for proper functioning or targeting of the protein. Several other ubiquitin like protein (Ubl) have been identified in eukaryotes, homologs for six of which have been found in *Plasmodium* including, Nedd8 (neural precursor cell expressed developmentally down-regulated 8) [138], small ubiquitin-related modifier (SUMO) [139], Hub1, ubiquitin-related modifier 1 (Urm1) [138], and autophagy-8 (Atg8) [140]. In *P. falciparum*, in silico studies identified four predicted sources of ubiquitin moieties. The polyubiquitin gene PFL0585w (contains five conserved ubiquitin repeats), two ubiquitin fusion proteins PfUBS27a and PfUBL40 (contain a ubiquitin moiety at their N-terminus) and PfsUb which is targeted to apicoplast [140–142]. Ubiquitin is removed by selective proteases known as Deubiquitinating proteases (DUBs) that selectively hydrolyse the isopeptide linkage. Two independent studies identified 18 and 29 Deubiquitinating enzymes (DUBs), respectively, in *Plasmodium* [139, 141]. DUBs are involved in removal of ubiquitin chain from proteins. Homologs of human UCH37, PfUCH54; and of UCHL3, PfUCHL3, are the only DUBs to be characterised so far and shown to exhibit deubiquitinating and deNeddylating activities [138, 143]. DUBs have been classified into at least five distinct subfamilies based on their sequence similarity and likely mechanisms of action including: (1) UBP (Ubiquitin specific processing protease). (2) OTU (Ovarian TUmour) related proteases. (3) UCH (Ubiquitin C-terminal hydrolases) and (4) Machado-Joseph disease protease (MJD). Of these, the first three are cysteine proteases, while the last one is a novel group of zinc-dependent metalloprotease. A bioinformatics approach to identify components of the ubiquitin mediated

pathway in apicomplexan parasites identified three OTU family proteins in *P. falciparum* [144]. Additionally, the presence of a functional Deubiquitinating enzyme PfUCH54 has been shown by [145]. The deNeddylating activities of PfUCH54 and PfUCHL3 might be of particular interest for drug design, since such activity is not known for the mammalian homologs of the two DUBs.

Protein modification by SUMO is also found in *P. falciparum*; however, its role in the regulation of the parasite life cycle is poorly understood. SUMOlyated proteins are widely distributed in parasite and PfSir2 is one of the targets along with several other putative targets [146]. Functional studies of a SUMO-specific protease (SENP) of *P. falciparum*, PfSENP1 demonstrated that this protease has unique cleavage sequence preference relative to the human SENPs [139]. In addition, a small molecule inhibitors of this protease can inhibit *P. falciparum* replication in infected human blood.

6.3 Signal and Transit Peptide Cleaving Proteases

To survive in the host erythrocyte the parasite has evolved a powerful protein secretion system responsible for trafficking of protein to sub-cellular organelles, host erythrocyte cytosol, host erythrocyte membrane, and secretion into the host milieu. The key player in secretion and trafficking of parasite proteins are the signal peptidase that are involved in cleaving the signal sequence from the target proteins, after this processing the proteins are released from the membrane and then routed to their destination. A total of five signal peptidases have been identified in *P. falciparum* that may be involved in formation of a signal peptidase complex (SPC) [5, 147, 148]. Another processing protease is Signal Peptide Peptidase that is an aspartyl family protease and is involved in cleavage of remnant signal peptides after their release by signal peptidase. The *P. falciparum* SPP was earlier suggested to be involved in invasion of merozoite by targeting a possible substrate, Band-3 [149, 150]; however later studies established its localization in parasite ER its role in growth of the asexual stage parasite [151, 152]. As mentioned above a number of nuclear encoded proteins are trafficked to mitochondria, apicoplast and other organelles in the parasite depending upon the presence of specific targeting sequences. Upon reaching the organelle membrane these transit peptide sequences are cleaved off from the proteins. Not many proteases are identified to be involved in processing of these transit sequences in different parasite organelles, only Falcilycin is implicated in processing of transit peptide in these organelles [78, 80].

To modify the host erythrocyte and to evade the host immune response, the parasite exports large number of proteins beyond the parasitophorous membrane. Most of these proteins contain an N-terminal signal sequence responsible for entry into ER as in case of other secreted proteins; in addition, these proteins also contain a motif termed PEXEL (*P*lasmodium *EX*port *EL*ement) which is responsible for trafficking of these proteins beyond parasitophorous membrane. The PEXEL motif is a pentameric sequence RxLxE/Q/D that is processed after the conserved leucine in

the ER and then gets N-aceylated, this cleavage is suggested to be essential for trafficking of target proteins beyond parasite boundaries [153, 154]. An aspartic protease Plasmepsin V which resides in ER is shown to be responsible for cleavage of PEXEL and facilitating trafficking of the proteins [155].

7 Conclusion

In view of the development of resistance of the parasite to frontline antimalarials, evolution of insecticide-resistant mosquitoes and unavailability of a vaccine, there is an urgent need to identify new drugs targets in the malaria parasite and develop novel anti-malarials, In the future, antimalarial therapy must combine several features that are still far from being ideal, including: minimal toxic side effects, high efficacy against resistant strains of *P. falciparum*, activity against several *Plasmodium* species, optimal pharmacokinetic profile, and low cost of therapy. Protease seems to be important players in lot of mechanism of survival for the parasite and thus seems to be wonderful target for developing new anti-malarials. Protease like Subtilisins and SERAs are good drug targets are they play essential roles in egress pathway of parasite; food vacuole protease will remain the best choice as drug targets as they are involved in hemoglobin degradation, one of most important pathways for parasite survival. Recent work on organelle proteases has also shown them to other plausible targets for drug development. There is need of combined effort from protein structural studies, computational biology and synthetic chemistry to develop more potent antimalarials.

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Proteases in *Vibrio cholerae* and Their Role in Pathogenesis

Amit Pal and Sun Nyunt Wai

Abstract Hemagglutinin protease (HAP) produced by *Vibrio cholerae* is the most well characterized protease in pathogenic Vibrio. The matured 45-kDa and 35-kDa processed forms of HAP were purified from a *ctx* gene negative *Vibrio cholerae* O1 strain. The 35-kDa HAP showed hemorrhagic fluid response in a dose dependent manner in the rabbit ileal loop assay (RIL). Histopthological examination of purified protease treated rabbit ileum showed the presence of erythrocytes and neutrophils in the upper part of the villous lamina propria, gross damage of the villous epithelium with inflammation, hemorrhage and necrosis. The 35-kDa form of HAP, when added to the luminal surface of the rat ileum loaded in an Ussing chamber, showed a decrease in the intestinal short-circuit current and a cell rounding effect on HeLa cells. Almost all results of earlier studies suggest an indirect pathogenic role of HAP but this study showed the possibility of a direct role of HAP in pathogenesis. The other well characterized protease secreted by *Vibrio cholerae* is PrtV a metalloprotease which has a role in the protection from predator grazing in natural aquatic environments and also has a role in human pathogenicity. PrtV also modulated hemolysin which plays a role in inflammatory response in human epithelial cells. A novel 59-kDa serine protease was identified from a Δ*hapA*Δ*prtV V. cholerae* O1 strain. The serine protease has been shown to cause hemorrhagic fluid response in RIL assay. Proteases secreted in *Vibrio cholerae* play a role in its pathogenesis.

Keywords *Vibrio cholerae* pathogenesis • Hemagglutinin protease (HAP) • *Vibrio cholerae* protease (PrtV) • 59-kDa Serine protease

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1 Introduction

The major features of pathogenesis of cholera are well established. Infection due to *Vibrio cholerae* begins with the ingestion of contaminated water or food. After passage through the acid barrier of the stomach, the organism colonizes the epithelium of the small intestine by means of the toxin-coregulated pili [1] and possibly other colonization factors such as the different hemagglutinins, accessory colonization factor and core-encoded pilus, all of which are thought to play a role. Cholera enterotoxin produced by the adherent *vibrios* is secreted across the bacterial outer membrane into the extracellular environment and disrupts ion transport by intestinal epithelial cells. The subsequent loss of water and electrolytes leads to severe diarrhea which is characteristic of cholera [2].

Proteases produced by pathogenic microorganisms play an important role in virulence [3]. Proteases are enzymes that catalyze the hydrolysis of peptide bonds in proteins or peptides. They are either exopeptidases, whose actions are restricted to the N- or C-termini of proteins, or endopeptidases which cleave internal peptide bonds. Microbial peptides are predominantly secreted enzymes and can be classified based on the essential catalytic residue at their active site. They include serine proteases, cysteine proteases, aspartate proteases and metalloproteases. Tissue barriers to pathogen invasion, such as extracellular matrices, epidermal keratinocyte layers and blood vessel walls, may be targeted by bacterial proteases. Proteolysis of host tissue components such as extracellular matrix proteins, including collagen, laminin, fibronectin and elastin, can induce necrotic tissue damage which may aid the bacteria in host cell entry [4, 5]. *V. cholerae* O1, the causative agent of epidemic cholera, secretes a 32-kDa zinc-containing hemagglutinin protease. Most results suggest that HAP plays an indirect role in pathogenesis. Our study is the first study to show direct role of HAP in pathogenesis of *V. cholerae* [6]. A metalloprotease, other than HAP, *Vibrio cholerae* protease (PrtV) plays a role in protection from predator grazing in natural aquatic environments. Using a Δ*hapA*Δ*prtV V. cholerae* strain a novel 59 kDa serine protease was identified and shown to play a role in hemorrhagic response in rabbit ileal loop [7].

2 Proteases in *Vibrio cholerae*

2.1 Hemagglutinin Protease

V. cholerae O1 El Tor biotype strains, as well as the non-O1, non-O139 serogroup strains produce several hemagglutinins [8–11] which may potentially be involved in adherence of the vibrios to the human gut. In particular, the soluble hemagglutinin, which also has proteolytic activity [12] is called hemagglutinin/protease (HAP). HAP (vibriolysin) belongs to a family of metalloprotese (M4: M04/003) which is largely distributed among pathogenic bacteria. Comparison of multiple

sequences shows considerable sequence similarity among primary structures of all known neutral proteases of M4 family. The active site and metal binding residues are highly conserved. HEXXH is the putative signature of Zn-metalloprotease. This motif contains all of the residues that have been positively identified as part of the active site [13, 14].

When HAP is purified in the presence of EDTA, a larger 45-kDa form is isolated while in absence of EDTA, a 32-kDa form is purified [6]. The protease undergoes several steps of processing, including cleavage of the signal peptide and further processing of the N terminus and the C terminus to generate the mature protein [14]. The 45-, 37-, 32-, and 9-kDa bands are the major bands bound to anti-HAP antibodies, which suggests that they are the proteolytic degradation products of the 45-kDa polypeptide [15]. Purified HAP in the absence of EDTA from *V. cholerae* non-O1, non-O139 strain PL-21 in TSB medium showed the presence of both 45- and 35-kDa forms [6]. In the presence of EDTA, the 45-kDa form of HAP was purified in larger amounts but in an in-active state. This is first study in which the two major forms of HAP were purified and studied in Ussing's chamber, rabbit ileal loop and tissue culture assays. The functional activity of HAP is related to its protease activity, and HAP being a metalloprotease, it shows no protease and HA activity in the presence of EDTA. The mature 45-kDa form of HAP purified in the presence of EDTA showed no effects in RIL, Ussing chamber, and tissue culture assays. Functional forms were generated when 45-kDa HAP was dialyzed in EDTA-free buffer. Once the protease is functional, it is processed, as is evident by the presence of 45, 42, and 35-kDa bands on SDS-polyacrylamide gels when HAP was purified in the absence of EDTA. *V. anguillarum* secretes a metalloprotease that also exists in two forms, Pa and Pb [16]. The Pb form was converted to the Pa form when the protease was incubated overnight at 4 °C. HAP exists in both the 45-kDa and 35-kDa forms but is not completely converted to the smaller processed form at 4 °C. Functional studies with only the 45-kDa form are not possible, as one cannot prevent its processing to the 35-kDa form in the absence of EDTA. However, the 45-kDa form can be studied through in vitro models such as Ussing chamber and tissue culture assays. This protein, at lower concentrations of 2 μg/ml and 4 μg/ml, exerts an enterotoxic response in the Ussing chamber in the form of an increase in I_{SC} (Fig. 1a) and a cell distending effect in the HeLa cell assay (Fig. 2b). At higher concentrations, it shows a decrease in the I_{SC} in the Ussing chamber (Fig. 1a), suggesting a cytotoxic response and a cell rounding effect on HeLa cells (Fig. 2a). The most probable explanation for this differential effect is that, at lower concentrations of functional 45-kDa HAP, the amount of 35-kDa form is less abundant, while the amount of the processed 35-kDa form is elevated at higher concentrations of 45-kDa HAP and may be higher than a critical level when it exerts a cytotoxic effect. The I_{SC} changes peaked at 4 μg/ml, while the effect observed at 6 μg/ml revealed that the enterotoxic response of the 45-kDa form was masked by the cytotoxic effect of the 35-kDa form, resulting in a decrease in I_{SC}. The enterotoxic response of the 45-kDa HAP started after a delay of 45–60 min and was equivalent to the effect of 1 μg/ml of CT in the rat small intestine [17]. Figure 1a shows that all protein concentrations of 45-kDa form upto 4 μg/ml the I_{SC} exhibited a gradual increase in

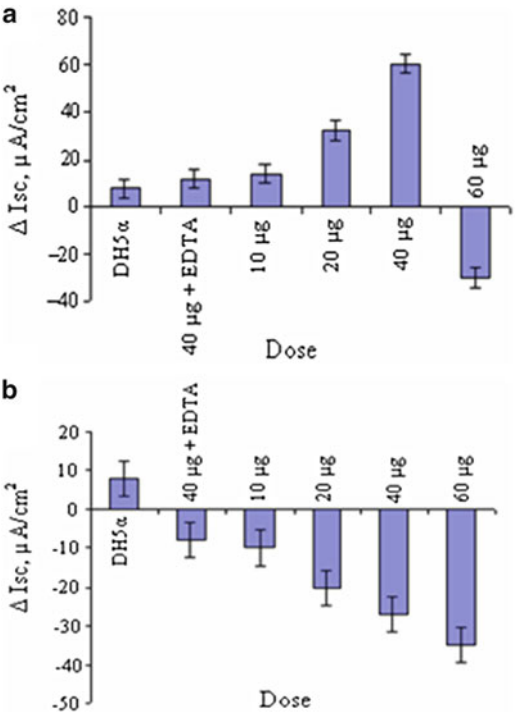

Fig. 1 Dose dependent response of HAP on intestinal short circuit current (Isc) of rat ileum loaded in Ussing's chamber. HAP was added in absence or presence of EDTA to the Ringer's solution in Ussing's chamber and the change in Isc was recorded for next 2 h from the time the sample was added. The data represents the mean and standard deviation for triplicate experiments. Proteins purified from DH5α were used as controls. (**a**). Response of Isc to increasing doses of 45-kDa HAP. (**b**). Response of Isc to increasing doses of 35-kDa HAP. There is an initial increase, but the Isc falls as the dose reaches beyond 40 μg/ml. Values are the mean standard deviations (error bars) for triplicate experiments. Proteins purified from E. coli DH5α were used as controls

rabbit ileal mucosa, with the peak being reached at different time intervals, depending on the protein concentration used, indicative of Cl^- secretion. The addition of glucose at the end of the experiment showed equivalent responses in treated and untreated tissue, indicating that glucose-Na^+ active co-transport function was unaffected. Enterotoxicity is caused by an abnormal flux of negatively charged ions from the serosal side to the mucosal side [18]. Interestingly, at a higher concentration (6 μg/ml), there was a significant decrease in I_{SC} (Fig. 1a). The dose dependent effect of the purified 35-kDa HAP showed a sharp decrease in the intestinal short circuit current (Fig. 1b). The purified 35-kDa form of HAP also showed cell rounding effect (Fig. 2a). It was earlier reported that culture supernatant prepared from the reactogenic strains of *V. cholerae* cause a decrease in the transcellular epithelial resistance of cultured T84 cells [19]. This decrease correlated with the presence of HAP but not with the presence of other potential accessory toxins or proteases [19].

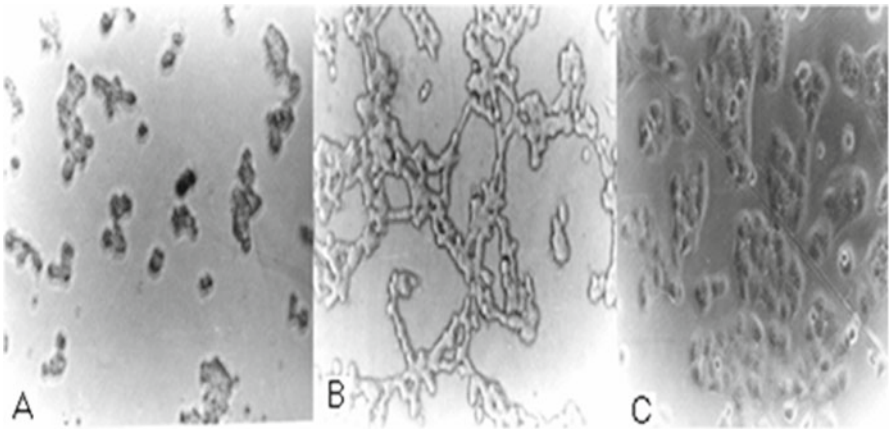

Fig. 2 Effects of HAP on HeLa cells. HeLa cells grown in tissue culture dishes were treated overnight with different concentrations of either the 35-kDa HAP or the 45-kDa HAP. Cells were observed under phase contrast microscope. (**a**). Cell rounding effect of 35-kDa HAP and higher concentration of 45-kDa HAP. (**b**). Cell distending effect of lower concentration of 45-kDa HAP (**c**). Normal HeLa cells grown in tissue culture plates

Purified HAP showed a dose-dependent hemorrhagic fluid response in the rabbit ileal loop assay. At 20 μg, a weak hemorrhagic response was elicited. At HAP concentrations increasing up to 60 μg there was a gradual increase in the fluid accumulation ratio, with hemorrhagic fluid present at all the different concentrations. The protease incubated in the presence of EDTA failed to show any response in RIL assay. The results of the RIL assay were substantiated by histopathological analysis of the ileal loop samples. In all the experiments, the rabbits were sacrificed 18 h postinoculation. The villous architecture and mucosa in the buffer control sections were normal (Fig. 3a). Purified HAP at a concentration of 20 μg resulted in little distortion of the villous pattern. The blood vessels were congested, and inflammatory cells were present in the lamina propia. High-powered photomicrograph showed the presence of erythrocytes in the upper part of the villous lamina propria. After treatment with 40 μg of purified HAP, there were marked alterations of the villous contour with red blood cell and inflammatory cell infiltrates in the villous epithelium and lamina propria and in damaged crypt epithelium (Fig. 3b). Higher magnification of portions of Fig. 3a, b revealed the clear presence of erythrocytes, eosinophils, and neutrophil polymorphs in the lamina propia (Fig. 3c, d). Washed *V. cholerae* 569B cultures, on the other hand, showed non-hemorrhagic fluid accumulation in the RIL assay. The active dialyzed 45-kDa form tested for entertoxigenicity in the RIL assay also showed hemorrhagic fluid response

HAP can proteolytically activate CT-A subunit [20] and the El Tor cytolysin/hemolysin [21] and can hydrolyze several physiologically important proteins such as mucin, fibronectin, and lactoferrin [22]. HAP perturbs the paracellular barrier of cultured intestinal epithelial cells [19, 23] by acting on tight-junction-associated proteins [23] and promotes the detachment of vibrios from monolayers and mucin [24, 25].

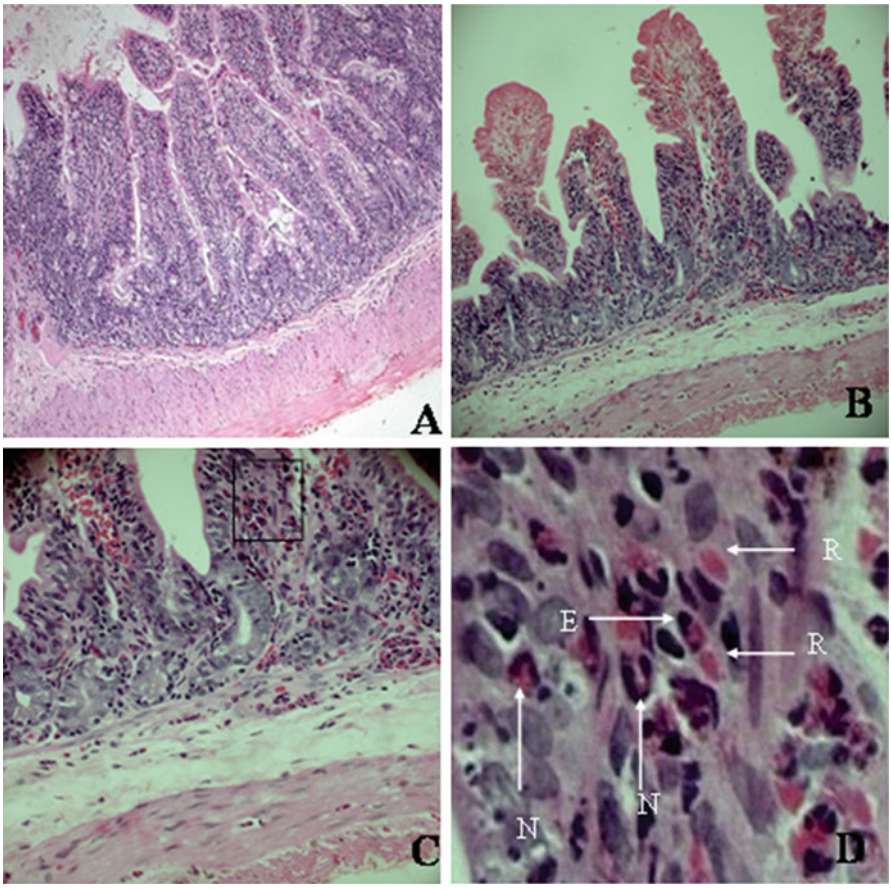

Fig. 3 Effect of purified HAP in the RIL assay. Purified HAP treated ileal tissues were processed for histopathological analysis and photomicrographs were taken. (**a**) Villus architecture observed in 25 mM Tris-HCl treated ileal tissues (10× magnification). Figure shows normal villous structure. (**b**) 40 μg of purified 35-kDa HAP treated rabbit ileal tissues show disruption of normal villus architecture with shortening of the villi (20× magnification). (**c**) 40× magnification of Fig. 4b shows infiltration of polymorphonuclear neutrophils, eosinophils and erythrocytes. (**d**) The *inset box* in Fig. 4c was further magnified (200×) to show the presence of RBC (*R*), neutrophils (*N*) and eosinophils (*E*). Magnification 200×

2.2 Vibrio cholerae *Protease (PrtV)*

The second most well characterized protease in *Vibrio cholerae* is the *Vibrio cholerae* protease (PrtV). Using reverse molecular genetics techniques an extracellular protease was identified as PrtV protein as being necessary for killing nematodes by *Vibrio cholerae* [26]. The killing effect is associated with colonization of the *C. elegans* intestine. The nematode *Caenorhabditis elegans* has been used successfully as an invertebrate model to screen for virulence factors of several human pathogens e.g. *P. aeruginosa* and *S. typhimurium* [27, 28]. *V. cholerae* lethal

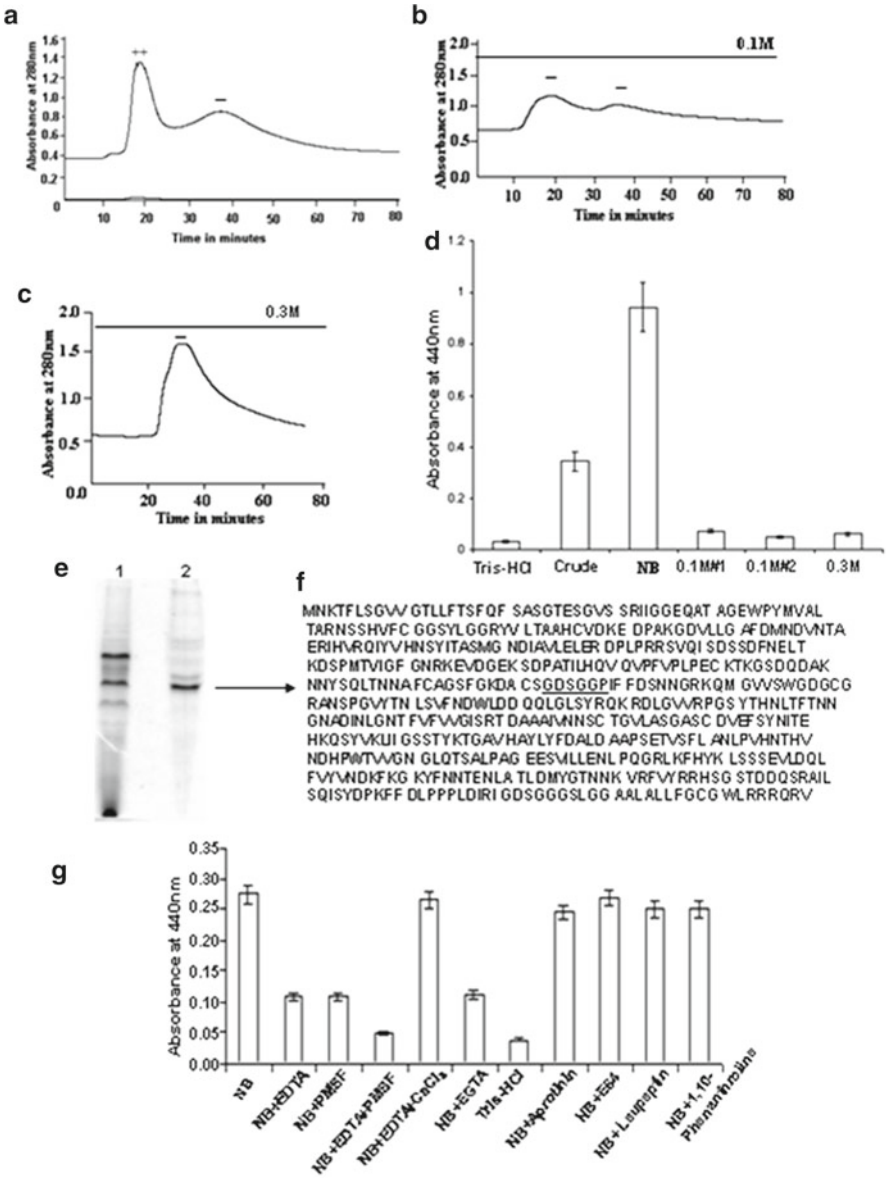

Fig. 4 Partial purification and identification of protease. Chromatographic profile of ammonium sulphate precipitated crude proteins from culture supernatants of CHA6.8Δ*prtV* strain loaded onto an anion exchange column (DE-52). (**a**) Proteins eluted in the non-binding fraction (NB), (**b**) proteins eluted with 0.1 M NaCl, (**c**) proteins eluted with 0.3 M NaCl, +/− shows presence or absence of protease activity, (**d**) azocasein assay with pooled samples (30 µg) NB, 0.1M#1, 0.1M#2, 0.3 M and crude proteins. (**e**) Native PAGE profile (*lane 1*) of crude proteins of CHA6.8Δ*prtV* strain and (*lane 2*) of partially purified protease (NB) from DE-52 column. The marked protein band was analyzed by MS/MS sequencing and the peptides *highlighted* showed homology with a 59-kDa trypsin-like serine protease encoded by VC1649. (**f**) The *underlined* GDSGGP are the amino acid sequences around the serine residue present in trypsin-like serine proteases. (**g**) Protease inhibition test of NB fraction (5 µg) with protease inhibitors 10 mM EDTA, 25 mM PMSF, 25 mM PMSF and 10 mM EDTA, 10 mM EDTA and 20 mM $CaCl_2$, 10 mM EGTA, 1 µg/ml aprotinin, 28 mM E64, 1 µg/ml leupeptin and 10 mM 1,10-phenanthroline incubated for 30 min at 37 °C. Residual protease activity was assayed by azocasein assay. Twenty-five mM Tris-HCl was used as a negative control. The values shown are the means with standard deviations from three experiments

infection of the *C. elegans* depends on a HapR-regulated protease. HAP encoded by *hapA* gene is regulated by *hapR*, and is the major extracellular protease. However, when *hap*A mutant *V. cholerae* was tested in *C. elegans* killing assay no attenuation was found in comparison with the wild type. The culture supernatant from *hapA* mutant contained some proteins not found in the supernatant of *hapR* mutant [26]. The three most abundant putative *hapR*-regulated proteins detected in the supernatants from the *hapA* mutant *V. cholerae* by mass spectrophotometer revealed that the protein bands corresponded to proteins encoded by the ORFs VCA0812, VCA0813 and VCA0223. The protein products are a leucine aminopeptidase-related protease, leucine amino aminopeptidase (Lap) [29] and the PrtV protease [30] respectively. Knock mutants Δ*prtV*, Δ*lap* and Δ*lapX* mutants were tested in *C. elegans* model and of all the mutants only Δ*prtV* mutant showed complete attenuation in comparison to wild type. Neither of the Δ*lap* and Δ*lapX* mutants showed significant attenuation. Furthermore, the cloned *prtV* gene resulted in an increased killing effect by the wild type *V. cholerae* strain C6706, and it restored a significant killing effect in the case of the *hapR* mutant derivative. PrtV is important for *V. cholerae* inhibition of natural Bacteriovorous predators. *V. cholerae* caused a marked reduction in the number of viable protozoa (*Cafeteria roenbergensis* and *Tetrahymena pyriformis*) during prolonged co-cultivation, whereas the bacterial density remained virtually unaltered. Furthermore *V. cholerae* with mutations abolishing the *hapR* or *prtV* genes appeared strongly attenuated in the predator grazing test, and most of the bacteria were consumed by the predator within 3 days. The metalloprotease PrtV was purified from the culture supernatants of a *Vibrio cholerae* derivative that is deficient in several other secreted peptidases, including the otherwise abundant hemagglutinin protease [31]. The PrtV is synthesized as a 102 kDa protein, but undergoes several N-and C-terminal processing steps during *V. cholerae* envelope translocation and prolonged incubation [31]. Purified *V. cholerae* PrtV protease forms of 81 or 73-kDa were stabilized by calcium ions. Removal of calcium resulted in further rapid autoproteolysis. In assay using cultured cells of the human intestinal cell line HCTS, the PrtV protein showed a cytotoxic effect leading to cell death. PrtV also degraded the extracellular matrix components fibronectin and fibrinogen. PrtV mutants were not attenuated in the infant mouse model, nor did they exhibit reduced colonization potential compared with wild type in competition experiments [30]. PrtV also modulates the response of *Vibrio cholerae* hemolysin in human intestinal epithelial cells. *Vibrio cholerae* hemolysin is capable of causing an inflammatory response characterized by increased permeability and production of IL-8 and TNF-alpha in tight monolayers. The inflammatory response was totally abolished by treatment with PrtV [32].

2.3 59-kDa Serine Protease

Although HAP is a very active virulence factor, an isogenic strain of *V. cholerae* mutated in the *hap* gene was no less virulent in infant rabbits than the parental strain [24].

An earlier study by Hase et al. [33] showed that a *hapA*-deleted mutant of *V. cholerae* O1 had reduced extracellular proteolytic activity compared with the parental strain in a skim milk assay, indicating that the mutant still produces some extracellular proteolytic activity. In addition, residual proteolytic activity expressed by the *hapA*-deleted mutant is distinct from HAP, as demonstrated by failure of anti-HAP serum to inhibit the activity of this secondary protease on milk agar. The mutant strain also failed to agglutinate chicken erythrocytes [24]. Young and Broadbent [34] described several extracellular proteases in *V. cholerae* that could explain the residual proteolytic activity of the *hap*-negative *V. cholerae* mutant. Besides HAP, the other major well-characterized protease in *V. cholerae* is a 97-kDa *Vibrio cholerae protease*, PrtV. PrtV plays a role in virulence in a *C. elegans* infection model [26].

A *hapA* and *prtV* double knock out mutant of *V. cholerae* strain CHA6.8Δ*prtV* still had residual protease activity [7]. This protease was partially purified from strain CHA6.8Δ*prtV*. The ammonium sulphate precipitated proteins from culture supernatants of CHA6.8Δ*prtV* were loaded onto an anion exchange chromatography column (DE-52) [7]. The proteins in the non-binding (NB) fraction of the column (Fig. 4a) were pooled and concentrated. The bound proteins were eluted with 0.1 M (Fig. 4b) and 0.3 M (Fig. 4c) NaCl, dialyzed against 25 mM Tris-HCl buffer and concentrated. When protease activity in the NB, 0.1 M and 0.3 M NaCl eluted fractions were tested by azocasein assay, the major protease activity was present in the NB fraction (Fig. 4d). The NB-pooled fraction was concentrated and run on a native PAGE (Fig. 4e). The major protein band was excised and analyzed by MS/MS sequencing (Fig. 4e). The sequences highlighted showed homology with a 59-kDa serine protease encoded by the gene VC1649 (Fig. 4f). The sequence GDSGGP (underlined) flanks the serine residue in trypsin-like serine proteases (Fig. 4f). To determine the nature of the partially purified protease from CHA6.8Δ*prtV* eluted in the non-binding fraction of a DE52 anion-exchange column, we performed protease inhibition assays with several inhibitors (Fig. 4g). The protease was partially inhibited in the presence of EDTA (60.3 %), EGTA (59.2 %) and PMSF (60.3 %). The partially purified protease was completely inhibited when PMSF and EDTA are used together (Fig. 4g). There was significantly less inhibition of protease activity in the presence of 1, 10-phenanthroline (9 %), aprotinin (10.5 %), leupeptin (8.7 %) and E64 (1.8 %). Although EDTA inhibited protease activity by 60.3 %, EDTA in the presence of $CaCl_2$ inhibited activity by only 3.6 % inhibition (Fig. 4g). The serine protease secreted byCHA6.8Δ*prtV* is a calcium-dependent serine protease.

To study the role of the 59-kDa serine protease in virulence, 50 μg of the partially purified protease was injected into the rabbit ileum, which induced significant hemorrhagic fluid accumulation (FA ratio 1.2+/20.2, n = 3, Fig. 5a). When a similar concentration of the protease was incubated in the presence of PMSF and EDTA and injected into the rabbit ileum, there was a significant decrease in fluid accumulation (FA ratio 0.3+/20.05, n = 3, Fig. 5a). Histopathological analysis of the rabbit ileum revealed that the protease caused extensive damage to all the layers of the mucosa. There was damage to the villus surface structure with hemorrhage in all layers of the mucosa. On the other hand, analysis of the ileal tissues treated with the

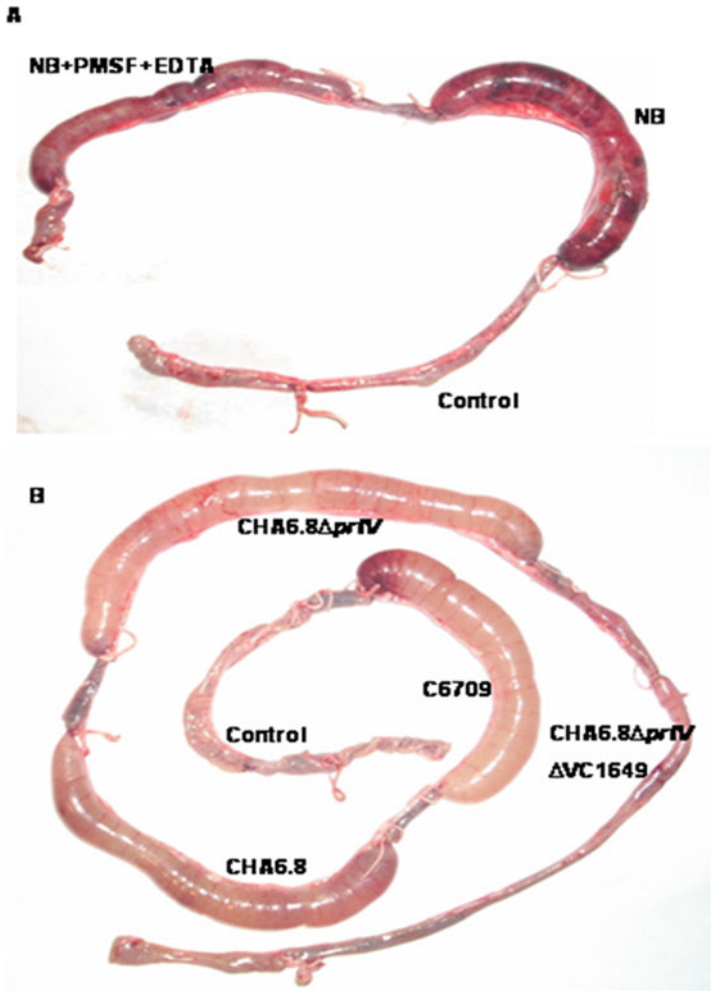

Fig. 5 Rabbit ileal loop assay. (**a**) RIL response of partially purified protease (50 μg, NB) showing significant hemorrhagic fluid accumulation (FA ratio 1.2 +/–0.2 n = 3) and its effect after inhibition with 25 mM PMSF and 10 mM EDTA (NB + PMSF + EDTA) shows significant decrease in fluid accumulation (FA ratio 0.3 +/–0.05 n = 3). Twenty five mM Tris-HCl with 25 mM PMSF + 10 mM EDTA was used as a negative control (FA ratio = 0.12 +/–0.002, n = 3). (**b**) RIL response with culture supernatants of C6709 (FA ratio 1.1 +/– 0.3, n = 3), CHA6.8 (FA ratio 1.08 +/– 0.2, n = 3), CHA6.8Δ*prtV* (FA ratio 1.02 +/– 0.2, n = 3), CHA6.8Δ*prtV*ΔVC1649 (FA ratio 0.11 +/– 0.005, n = 3) and Tryptic soy broth as negative control (FA ratio 0.09 +/– 0.002, n = 3)

protease in presence of both PMSF and EDTA, revealed normal microvillus structure with no gross alteration in villus structure, although the villus lamina propria was slightly dilated and RBCs had accumulated in a few places in the basal area. PMSF and EDTA completely inhibited protease activity (Fig. 5a), but still we observed some residual effect in the rabbit ileal loop. This effect could be due to some other domain in the protease, which may not be its proteolytic domain, and

could be responsible for causing damage to the ileal tissue. Tissues treated with 25 mM Tris-HCl and PMSF + EDTA did not cause fluid accumulation in RIL (FA ratio 0.12+/20.002 n = 3, Fig. 5a)

Rabbit ileal loop assay with one ml of culture supernatant of C6709, CHA6.8 and CHA6.8Δ*prtV* strain induced significant fluid accumulation (Fig. 5b) in RIL model (FA ratio 1.1+/20.3, n = 3; 1.08+/20.2, n = 3; and 1.02+/20.2, n = 3 respectively) where as CHA6.8ΔprtVΔVC1649 strain which is devoid of the serine protease gene and sterile tryptic soy broth, TSB (negative control) did not induce any fluid accumulation (Fig. 5b) (FA ratio 0.11+/20.005, n = 3 and 0.09+/20.002, n = 3 respectively). Almost similar results were observed when one ml of washed bacterial cells (10^9 cfu/ml) of C6709, CHA6.8 and CHA6.8Δ*prtV* induced significant fluid accumulation (FA ratio 1.2+/20.35, n = 3; 1.1+/20.3, n = 3; and 1.0+/20.2, n = 3 respectively). Bacterial cells of CHA6.8Δ*prtV*Δ*VC1649* strain did not induce any fluid accumulation (FA ratio 0.15+/20.005, n = 3). Ileal tissues treated with culture supernatant of CHA6.8Δ*prtV* strain also showed dilated villi with gross hemorrhage in all layers of the mucosa. The ileal tissues treated with culture supernatant of CHA6.8Δ*prtV*Δ*VC1649* strain protease showed villous architecture almost normal with minimum hemorrhage in mucosa and sub-mucosa.

V. vulnificus also secretes a 59-kDa serine protease, which is the free form, while the 69-kDa protein may be a complex form associated non-covalently with small peptide(s) [35]. The proteolytic activity of the final preparation was almost completely abolished by treatment with 5 mM PMSF, a well-known inhibitor of serine proteases. By contrast, tetraethylenepentamine, a specific inhibitor of metalloproteases including VVP (*Vibrio vulnificus* protease), showed no inhibitory effect on the proteolytic activity. *Vibrio vulnificus* strain NCIMB 2137, in which *vvp* has been deleted, secretes a serine protease, VVA0302. *V. vulnificus* serine protease may be a virulence factor in vibriosis, which is characterized by external and internal hemorrhages affecting the major organs [36], or human wound infection with necrotic tissue damage [37].

The proteomic analysis of the *V. cholerae* type II secretome revealed the presence of three related serine proteases named as (ves: vibrio extracellular serine proteases) *vesA*, *vesB* and *vesC* [38]. Experiments suggest that *vesA*, *vesB* and *vesC* do not contribute to the intestinal survival and colonization capability of *V. cholerae* in mice. VesA has shown to be responsible for processing the A subunit of cholera toxin during in-vitro growth of *V. cholerae* strain N 16961. *VesC* encoded by VC1649 (59-kDa serine protease) is not capable of processing cholera toxin under tested conditions but has been shown by us to play a role in hemorrhagic response in rabbit ileal loop and it may play a role in pathogenesis [7].

3 Leucine Aminopeptidase

The aminopeptidases (APs) form a group of zinc-dependent metalloproteases that catalyze the removal of amino acids from the N terminus of a protein. These enzymes are of biological and medical importance because of their role in protein

degradation and in the metabolism of biologically active peptides. Leucine APs (LAPs) preferably cleave leucyl substrates, although substantial rates of enzymatic cleavage with most amino-terminal residues are observed [39]. The structural gene for the *Vibrio cholerae* leucine aminopeptidase (lap) has been cloned and sequenced [29]. The molecular weight of LAP is 54.4 kDa. The deduced amino acid sequence of the entire protein showed high homology with the sequence of *Vibrio proteolyticus* leucine aminopeptidase. The protease showed maximum activity at pH 9.0 and was thermostable at 70 °C. The substrate leucyl-p-nitroanilide was cleaved by the protease, and its activity was inhibited by EDTA and bestatin. LAP may play an important role in releasing the free amino acids needed for the regulation of expression of other virulence factors [40].

4 Conclusions

V. cholerae, secretes several proteases like HAP, PrtV and 59-kDa serine protease. The major protease secreted by *V. cholerae* strains is the HAP. Almost all results suggest indirect pathogenic roles of HAP, the possibility of a direct role is also reported. Ghosh et al. [6] assayed the enterotoxic activity of HAP purified from a cholera toxin-negative *V. cholerae* strain. The purified HAP showed a positive hemorrhagic fluid response in the rabbit ileal loop assay and an increase in the intestinal short circuit current in the Ussing chamber assay. A metalloprotease, other than HAP, PrtV is thought to play a role in aquatic environment. Vaitkevicius et al. [26] showed the effect of PrtV against predator grazing in aquatic environment. PrtV also plays a role in pathogenicity of *V. cholerae*. The serine protease from a Δ*hapA*Δ*prtV V. cholerae* O1 strain induced hemorrhagic response in rabbit ileal loop [7]. Role of proteases in pathogenicity in *V. cholerae* could also depend on the expression levels of proteases. In an earlier study by Young and Broadbent [34], 100 strains of *V. cholerae* El Tor from different parts of the world were screened for protease production by a rapid assay with gelatin agar plates. Based on protease production, the strains were classified as high, medium and low protease producers. The expression of these proteases may play a role in pathogenicity of *V. cholerae* strains. Our results suggest that the expression of proteases in *V. cholerae* may follow a cascade of events. HAP controls secretion of the 59-kDa serine protease, which in turn may control secretion of other proteases.

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ADAMTS13: The von Willebrand Factor Cleaving Protease and Its Role in Thrombotic Thrombocytopenic Purpura

Surbhi Saini, Tal Schiller, Andrew Wu, and Chava Kimchi-Sarfaty

Abstract Since the discovery of ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) in the twentieth century, significant advancements have been made in understanding its role in hemostasis, molecular structure, genetics and genotype-phenotype relationships. It is a member of the ADAMTS family of matrix proteases and is responsible for the cleavage of ultra-large molecules of von Willebrand factor (VWF), thus regulating the adhesion of platelets to VWF multimers. Structurally, it resembles other members of the ADAMTS family with the exception of the number of thrombospondin-1 repeats and the presence of two CUB domains at the carboxyl terminal. The proteolytic activity of ADAMTS13 is mediated via an adamalysin-like metalloprotease domain. The *ADAMTS13* gene was cloned in 2001 and since then a number of disease-causing mutations have been discovered across the entirety of this gene. Additionally, ten different splicing isoforms have been described for *ADAMTS13* gene and a significant amount of genetic variations in this gene arises from the 1000 genome project (single nucleotide polymorphisms (SNPs)). Phylogenetic origins of ADAMTS13 are also discussed. Deficiency of this necessary protease activity due to autosomal recessive mutations of the *ADAMTS13* gene are implicated in congenital thrombotic

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thrombocytopenic purpura, also called Upshaw-Schulman syndrome. Ongoing research focuses on the role of ADAMTS13 in other forms of thrombotic microangiopathy and the development of either a plasma-derived or recombinant form of ADAMTS13 for therapeutic purposes.

Keywords ADAMTS13 • Thrombotic thrombocytopenic purpura (TTP) • Genomics • von Willebrand factor cleaving protease • Protein domains • Thrombotic microangiopathy

1 Introduction

The discovery of ADAMTS13 and its crucial role in maintaining hemostasis is one of the most insightful scientific stories of the past century. In 1924, Eli Moschcowitz described a 16-year-old girl with fever, petechia, acute renal failure, and paralysis. Postmortem histology revealed hyaline platelet thrombi in almost every organ [1]. Years later Schulman et al. and Upshaw reported an 8-year-old girl and a 29-year-old woman, respectively, who had chronic relapsing thrombocytopenia associated with microangiopathic hemolytic anemia and responded to plasma infusions [2, 3]. These cases established the disease entity henceforth called hereditary thrombotic thrombocytopenic purpura (TTP) or Upshaw-Schulman syndrome (OMIM number 274150). In 1982, Moake et al. were successful in showing that the plasma of patients with chronic relapsing thrombocytopenia contained unusually large factor VIII-VWF (von Willebrand factor) multimers and proposed that these patients lacked a 'large factor VIII-VWF depolymerase' [4]. Later, it became known that this protease was essential in breaking up the VWF multimers and preventing widespread, uncontrolled platelet aggregation and micro-thrombi formation. In the 1990s, several advances were made in understanding the physiologic activity and characterization of this plasma protease [5, 6] and in 1997, Furlan et al. showed that this von Willebrand factor cleaving protease (VWF-CP) was deficient in the plasma of a patient with chronic relapsing TTP [7]. The turn of the century saw significant breakthroughs in understanding the genomics of this protease when Levy and colleagues studied four families with congenital TTP and identified a new member of the ADAMTS family of zinc metalloproteases, naming it ADAMTS13. They also reported mutations in this gene to be responsible for absence of ADAMTS13 activity in these patients [8]. Almost concurrently, Zheng et al. cloned the cDNA, deduced the protein structure and domain organization and found out that alternative splicing is responsible for at least seven different variants [9]. In the past few years we have witnessed massive advances in elucidating the structural domains, functional nuances, immunogenicity, and genotype-phenotype relationships of the *ADAMTS13* gene and its protein.

In this book chapter, we aim to familiarize the reader with a basic overview of the structure of ADAMTS13, its function as the VWF cleaving protease and its significance in health and disease.

2 The Genomics of *ADAMTS13*

2.1 The ADAMTS13 Gene and Comparative Genomics

The gene, which has been assigned the symbol *ADAMTS13* by the HUGO Gene Nomenclature Committee, was mapped to 9q34.2. Within the phylogenetic tree, it has 54 orthologs (genes in different species that originated from a common ancestral gene and evolved during speciation) and 9 paralogs (genes related to each other by duplication within the genome; function of the genes may vary). Figure 1 traces

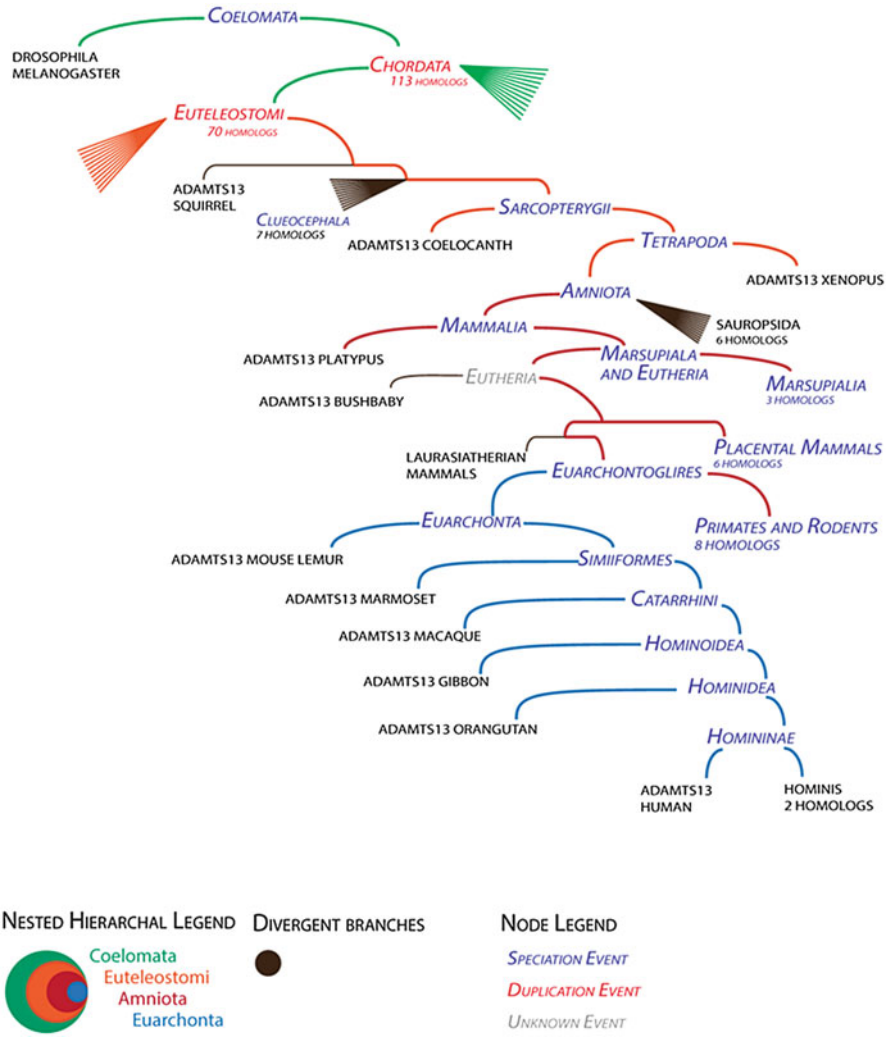

Fig. 1 *ADAMTS13* gene orthologs and maximum likelihood phylogenetic tree representing the evolutionary history of this gene. Evolutionary nodes are *italicized*: speciation events are indicated in *blue*; duplication events are indicated in *red*; unknown events are indicated in *gray*. Cladistic groupings are organized in a hierarchal manner

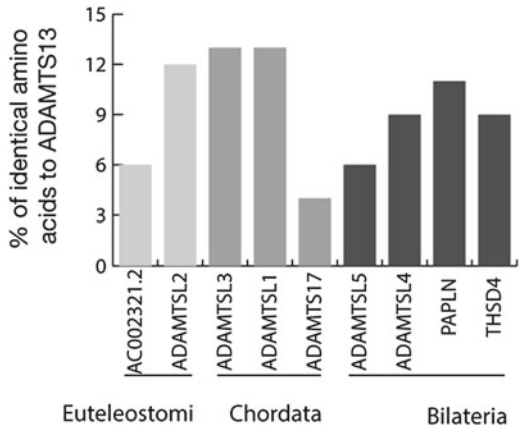

Fig. 2 In the X-axis, nine known paralogs of *ADAMTS13* gene are grouped per ancestral taxonomy. Y-axis gives the percentage of amino acids that are identical between *ADAMTS13* and a given paralog

the orthologs and Fig. 2 describes the paralogs in further detail based on the information provided in http://useast.ensembl.org/Homo_sapiens/Gene/Compara?db=core;g=ENSG000001603 23; r=9:136279478-136324508. This information may be useful when assessing the significance of polymorphisms and identifying new interactions or targets for ADAMTS13 functions.

The *ADAMTS13* gene is 45,049 base pairs (bp) long and lies between the 134,279,459–138,324,508 bp [NC_000009.11 Reference GRCh37.p9 Primary Assembly]. The open reading frame contains 29 exons and 28 introns which ultimately cipher for a 1,427 amino acid long protein chain. There are about 20 regulatory elements located in the region of the *ADAMTS13* gene and include promoter regions, transcription binding sites, and enhancers. Table 1 describes several highly relevant transcription factors of *ADAMTS13* gene.

2.2 *Disease-Causing Mutations in the ADAMTS13 Gene*

As of September 2012, up to 118 different mutations of the *ADAMTS13* gene have been reported in the literature. Of these, only 10 (9.8 %) occur in intronic regions, the rest being mutations in exons. These mutations include missense (63 %), nonsense mutations (9.8 %), splice site variants (8.8 %), nucleotide insertions (3.9 %), and deletions (15.7 %). More than half of these mutations (60 %) occur in the highly conserved N-terminal region that spans from the propeptide to the spacer domain and includes the catalytic metalloprotease domain. Figure 3 shows the distribution of unique mutations in the various regions.

2.3 *Single Nucleotide Polymorphisms in ADAMTS13*

An important part of the genetic variation in the *ADAMTS13* gene arises from single nucleotide polymorphisms (SNPs). Traditionally, SNPs are considered allelic variants

Table 1 List of relevant transcription factors of the *ADAMTS13* gene along with their binding positions and binding sequences (per NCBI Homo sapiens Build Number 37 Version 1)

Transcription factor	Family	Binding position	Strand	Binding sequence
FOXD1	Forkhead box	chr9:136287434-136287450	+	CCAAGAGTAAACACTGC
ER-alpha	Estrogen receptor	chr9: 136291147-136291166	+	TCCTGGCCATGCTGACCTGG
STAT5A	Signal transducer and activator of transcription	chr9: 136272970-136272994	+	CTTCTCTGAAAGTAGTCTCAGAGAC
HOXA5	Homeobox	chr9: 136277547-136277577	+	ATGCTCTCCTCCCCCTTAGGGCCCACGCCCA
AhR	Aryl hydrocarbon receptor	chr9: 136276115-136276131	+	TTATGCAGAGCGTGCCC
FOXF2	Forkhead box	chr9: 136287434-136287450	+	CCAAGAGTAAACACTGC
AREB6	Zinc-finger binding homeobox	chr9: 136290693-136290705	+	GGTGCACCTGGTG

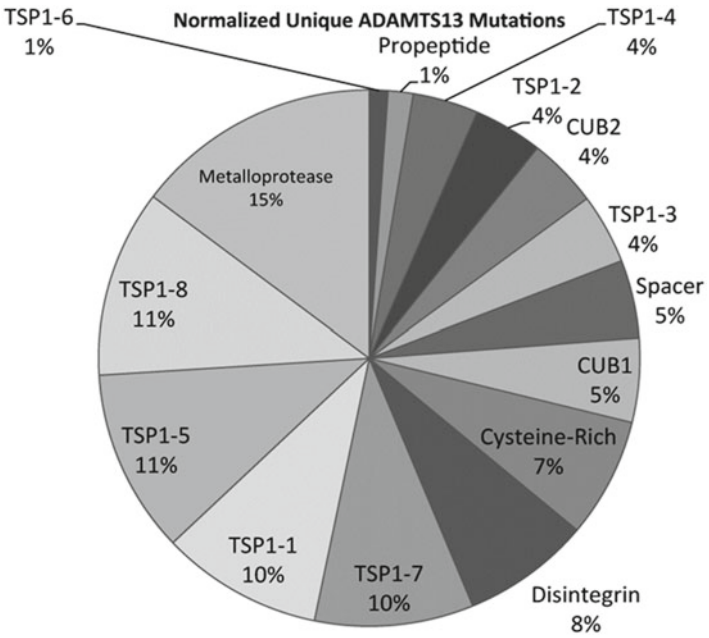

Fig. 3 Diagrammatic representation of the reported unique mutations per each domain of ADAMTS13

that occur with a frequency of >1 % in a given population. They may be located within coding regions and be synonymous (produce the same amino acid), or nonsynonymous (change in amino acid), or occur within non-coding regions and may still affect the expression of the gene by influencing splicing, transcription factor binding, microRNA, or the sequence of non-coding RNAs. As of September 2012, the number of variations reported by the National Center for Biotechnology Information's database [http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=11093; Contig label GRCh37.p5] reached 179 exonic (69 synonymous and 110 non-synonymous) and 672 intronic variations. Out of these, only 24 exonic (12 non-synonymous and 12 synonymous) and 176 intronic variations occur with a frequency of >1 % in a given population according to the 1000 Genomes Project sequencing database. Previous studies suggest that both synonymous and non-synonymous variations have an effect on the expression and specific activity of ADAMTS13, supporting observations about the wide range of protease activity in the normal population [10, 11].

2.4 Splicing Forms of ADAMTS13

Alternative pre-mRNA splicing is a post-transcriptional mechanism that varies among tissues and may also enrich the variations of the protein in different tissues [12]. It is believed that anomalous splicing may be responsible for numerous disease-causing

mutations as well [13, 14]. Ten different splicing isoforms have been described for the *ADAMTS13* gene; most were not fully characterized and not all produce a functional protein. Pre-mRNA splicing may account for ADAMTS13 protein size disparities between tissues; placental and skeletal muscle-derived *ADAMTS13* mRNA is approximately 2.4 kb long, while hepatic stellate-derived *ADAMTS13* mRNA is 3.7 kb long. Splicing isoforms have also been known to be specific to the prostate and brain. Our group described a unique splice form, missing most of the two CUB domains with additional intronic sequence, which is present in stellate liver cell cultures as well as several cancer cell lines. The protein was shown to be unsecreted [15].

3 Protein Structure of ADAMTS13

3.1 ADAMTS13 Protein Domains

ADAMTS13 belongs to the ADAMTS family of matrix proteases whose main function is extracellular matrix degradation and proteolysis of cell surface and soluble proteins [16]. They are characterized by having two unique regions: an N-terminus catalytic region containing disintegrin and metalloprotease domains and a C-terminus region that has thrombospondin Type I repeats. ADAMTS13 has the same skeleton as the rest of the ADAMTS family members but differs in the number of TSP1 repeats and the presence of two C-terminal CUB domains [17]. Figure 4 is a schematic representation of the ADAMTS13 protein with its various domains.

The ADAMTS13 protein sequence contains 1,427 amino acid residues with a calculated molecular weight of approximately 150 kDa. This precursor protein consists of a signal peptide (SP), a propeptide (P), a adamalysin-type metalloprotease (M) domain, a disintegrin-like (D) domain, a thrombospondin type 1 repeat (TSP1-1), a cysteine-rich (C) region, a spacer domain (S), a combination of TSPs (TSP1-2–TSP1-8), and two CUB (*c*omplement components C1r**C**1s/*u*rinary epidermal growth factor/*b*one morphogenetic protein-1) domains. Compared to other members of the ADAMTS family, ADAMTS13 has an abnormally short propeptide of ~41 residues. This propeptide is cleaved by furin, a proprotein convertase, at a specified furin cleavage consensus motif (RQRR). Interestingly, we found that a positively charged amino acid cluster (R-Q-R-Q-R-Q-R-R) present in the ADAMTS13 propeptide may act as a nuclear localization signal (NLS) [18].

In the mature form of the protease, the propeptide is followed by the adamalysin-like metalloprotease domain which spans 207 amino acid residues (position 80–286). This section of the protein is structurally and functionally related to the ADAM enzymes and matrix metalloprotease. The adamalysin activity requires binding of divalent molecules i.e. Zn^{++} and Ca^{++}. The adamalysin-like metalloprotease domain has three histidine residues (position 224, 228 and 234) that provide binding sites for the divalent cation Zn^{++}. In the 3-D structure solved part of the protein, Glu^{83}, Asp^{173}, Cys^{281} and Asp^{284} coordinate to form a low affinity Ca^{++} binding site, and there is evidence to show that another site predicted to involve residues Asp^{187} and Glu^{212} in conjunction with Asp^{182} or Glu^{184} may serve as a high affinity binding site for Ca^{++} as well [19].

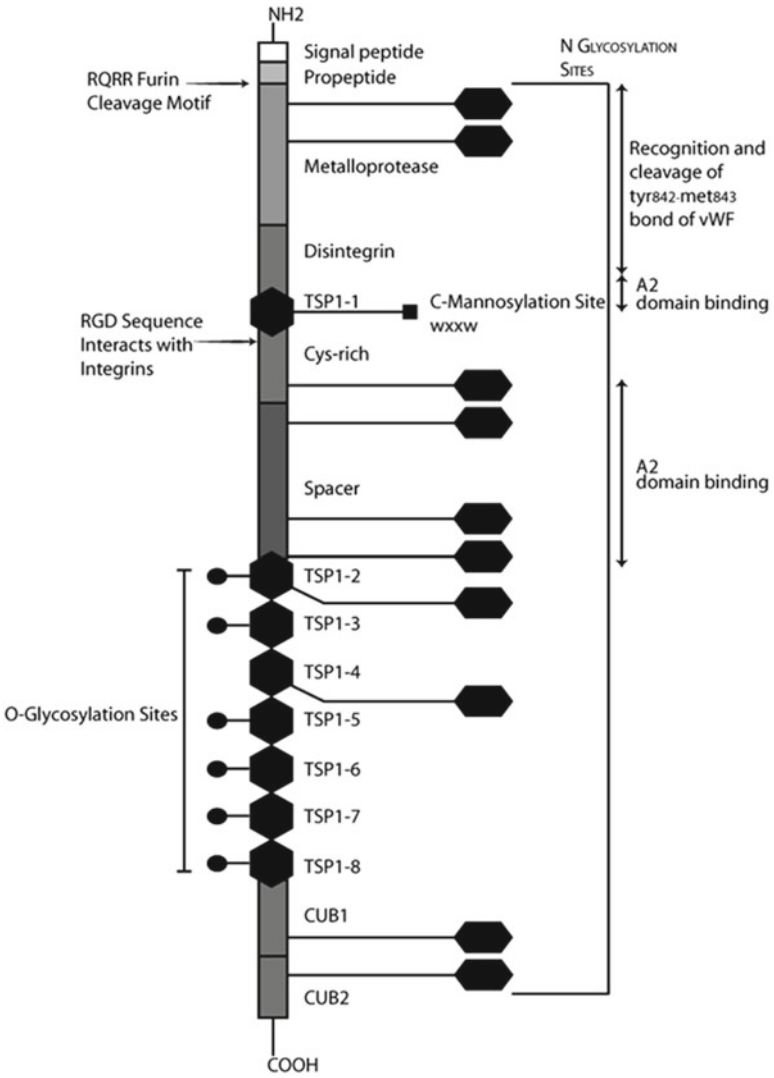

Fig. 4 Diagrammatic representation of the structure of ADAMTS13 protein, with details of the domains, binding sites and sites of post translational modification

Topographically, the metalloprotease is followed by the disintegrin-like domain which spans 97 residues from position 287–383. However, elucidation of the crystal structure of ADAMTS1 reveals that the disintegrin-like domain is functionally a part of the metalloprotease [20]. This is supported by demonstrating that the domain contains an exosite that binds the A2 domain of von Willebrand Factor, which harbors the substrate site of ADAMTS13 [21]. The first thrombospondin type 1 repeat follows the disintegrin domain and repeats 2–8 are spread between the spacer and CUB domains. All eight TSP-1 repeats are approximately 60 amino acids in length and contain

conserved Cys, Trp, Ser and Arg residues [22]. The presence of a total of eight TSP-1 repeats is unique to ADAMTS13 within the ADAMTS family. TSP-1 is a matricellular protein involved in angiogenesis, cancer, and inflammation [23]. The distinctive cysteine-rich domain follows next: it is 75 amino acid residues long and contains the typical Arg-Gly-Asp (RGD) sequence that provides the integrin binding site for platelets and other cells [24]. Multiple domains of ADAMTS13, particularly the cysteine-rich and spacer domains, are frequently targeted by anti-ADAMTS13 IgG antibodies in patients with acquired (idiopathic) TTP; however, a detailed discussion of acquired TTP is beyond the scope of this book chapter. The 130 amino acid long spacer domain is located downstream to the cysteine-rich domain and plays a critical role in substrate recognition during the proteolysis of VWF [25]. Another distinctive feature is the presence of two tandem CUB domains after the TSP1-8 domains. These sequences were first identified in the complement factor C1. The ADAMTS13 CUB-1 and CUB-2 domains contain five and two cysteine residues, respectively, and each of these residues differentially regulate the function of the metalloprotease [26]. Interestingly, although these domains do not exist in members of other ADAMTS family, mutations in these domains impair the secretion and function of the protease [15, 26].

3.2 Post-translational Modifications of ADAMTS13

Purified human plasma-derived ADAMTS13 has an observed weight of 190 kDa, higher than the calculated polypeptide weight of 150 kDa. Most of this additional weight comes from post-translational modifications. There are ten N-glycosylation sites spread across the entire metalloprotease and these post-translational modifications are important for its secretion and facilitate proteolysis of VWF [27]. In addition, TSP1-1 has the distinctive WXXW sequence that serves as a recognition motif for the attachment of an alpha-mannosyl residue to the C-2 atom of the first tryptophan, also known as C-mannosylation [22]. Six TSP1 repeats have the conserved sequence CSX(S/T) CG in which an O-linked dissacharide, Glc-Fuc, modifies the hydroamino acid at position 4 of serine or threonine, called O-fucosylation. The O-fucosylation has been shown to play a role in the secretion of ADAMTS13 [28]. Cis-trans isomerization of prolines in ADAMTS13 was shown by our group to be an additional post-translational modification important for the secretion: we have demonstrated that cyclosporin A significantly reduces secreted ADAMTS13 protein in vitro through impairing the activity of cyclophilin B [29].

4 ADAMTS13 Protein Function

4.1 Secretion

Circulating ADAMTS13 is constitutively active and plays a vital role in vascular hemostasis. It is produced by hepatic stellate cells [30], endothelial cells [31], and renal podocytes [32]. Recent reports suggest that platelets also have an intracellular

pool of ADAMTS13, which is functionally active and cleaves the ultra large VWF multimers (ULVWF) under flow as well as static conditions in vitro. Platelet-derived ADAMTS13 is present on the surface of the platelets and is stimulated by thrombin receptor activated peptide [33]. Moreover, almost 3 % of the plasma ADAMTS13 is bound to globular, plasma VWF, which facilitates the transport of the complex to areas of endothelial damage and ultimate incorporation into a platelet rich thrombus at the site of injury [34].

4.2 Proteolysis

ADAMTS13 cleaves VWF at the peptide bond between the amino acids Tyr1605-Met1606 (Tyr842-Met843 in the mature form) in the A2 domain of VWF. Under physiologic conditions, this scissile bond lays deeply buried within the core β-sheet of the globular conformation [35]. High flow shear stress, presence of denaturing agents, and platelet binding exposes this cleavage site to the metalloprotease, significantly increasing proteolysis. The precise regulatory mechanisms of this cleavage process still remains unknown, but under normal physiologic conditions, the proteolytic activity of ADAMTS13 is mainly regulated by the availability of the substrate and the specificity of the enzyme-substrate interaction. There are several factors that influence this cleavage by different mechanisms and include free hemoglobin >2 g/L, interleukin-6, tumor necrosis factor, thrombin, Factor Xa, and plasmin [36–38]. With normal plasma levels of ADAMTS13, this cleavage takes only seconds to minutes. The nascent VWF multimer is broken down into 176 and 140 kDa fragments that are normally found in circulation.

5 Pathophysiology of ADAMTS13 Deficiency

The absence or decreased activity of ADAMTS13 leads to the persistence of ULVWF in the circulation and may have significant clinical implications as will be discussed below. Multimeric VWF forms high strength bonds with the GP1b-IX platelet receptors under static as well as high fluid shear stress conditions. It is also believed that the multimer provides a higher number of platelet binding sites than its cleaved counterpart. Together, these phenomena are responsible for uncleaved VWF to bind avidly to more platelets, generating microthrombi that precipitate vessel occlusion and microangiopathic hemolysis. The aggregated platelets are in turn stimulated and release proinflammatory cytokines that add to the vessel wall injury, leading to a vicious cycle. An imbalance between VWF and ADAMTS13 has also been implicated in acute and chronic inflammatory states like SIRS (systemic inflammatory response syndrome), strokes, and coronary artery disease. As VWF is an acute phase reactant, its plasma levels rise in inflammatory states. At the same time, the plasma levels of ADAMTS13 are known to decrease, presumably due to

down regulation at the transcriptional level, proteolytic degradation, and excessive consumption. This leads to the persistence of ULVWF circulating in the plasma and might function as a biomarker of prothrombotic potential in inflammatory states [39].

6 ADAMTS13 Activity Assay Methods

Various tests have been developed to measure ADAMTS13 activity and expression (Table 2) in vitro.

Table 2 Assays to determine ADAMTS13 activity and ADAMTS13 antigen levels

Assay type	Assay description	References
SDS-Agarose Gel Electrophoresis and Western Blotting	Purified wild-type or mutant VWF with modified A2 domain is used to measure the proteolytic activity of plasma ADAMTS13. Plasma sample is incubated with purified VWF leading to formation of smaller multimers of the VWF. These smaller fragments are visualized using agarose gel electrophoresis followed by Western blotting with a specific peroxidase-conjugated anti-human VWF antibody. This method is complex and time consuming.	[5]
SDS-PAGE and Western Blotting	Purified VWF is used to measure the proteolytic activity of plasma ADAMTS13. This method is similar to the SDS-Agarose Gel Electrophoresis except that SDS-PAGE and Western Blotting allows direct visualization of the dimeric VWF fragments of 176 and 140 kDa. Technically easier than SDS-Agarose Gel Electrophoresis and sensitive.	[6]
FRETS VWF73 Assay	Based upon the fluorescence resonance energy transfer. This method uses chemically modified VWF73 containing the central A2 domain which is readily cleaved by normal plasma but not by ADAMTS13-deficient plasma. This cleavage releases fluorescence that is measured spectrometrically. This method is efficient, sensitive and reproducible.	[59]
Collagen-Binding assay	Based upon the principle that large VWF multimers preferentially bind to type III collagen. Purified VWF is incubated with test plasma in the presence of denaturing agents, leading to its cleavage. The residual VWF is then bound to collagen type III and a peroxidase-conjugated rabbit antibody against human VWF enzyme linked immunosorbent assay (ELISA) is used to quantify it. ADAMTS13 levels are inversely proportional to the quantity of collagen bound VWF. Results vary based on the collagen type, but are quite reproducible with type III collagen.	[60]
Ristocetin-induced aggregation	Based upon detection of ristocetin cofactor activity. Ristocetin-induced platelet aggregation (RIPA) is an ex vivo assay for live platelet function to aggregate with help of von Willebrand factor and exogenous antibiotic ristocetin added in a graded fashion Purified VWF is digested with the ADAMTS13 of the test plasma and the residual VWF is calculated by determining the ristocetin cofactor activity using a platelet aggregometer.	[61]

(continued)

Table 2 (continued)

Assay type	Assay description	References
Flow based assays	Based upon measuring ADAMTS13 activity under flow conditions which mimic physiologic fluid shear stress related cleavage of ULVWF more closely than static conditions.	[62]
ELISA	Uses epitope tagged recombinant fragments, either VWF73 or VWFA2, as substrates. ADAMTS13 monoclonal or polyclonal antibodies can be used. Recombinant VWF fragment, immobilized onto an ELISA plate with an antibody to one tag, is incubated with the test plasma sample. An antibody to another tag measures the residual substrate concentration and the ADAMTS13 activity is inversely proportional to the residual substrate concentration.	[63, 64]
Impact-R method	This test is relatively simple and sensitive. Based upon measuring platelet adhesion as determined by the percent of the well surface covered with platelets.	[65]
Vortex-based assay	ADAMTS-13 activity of test plasma is measured by the collagen-binding assay. Normal (type O) whole blood is mixed with test plasma (3:1) and then subjected to Impact-R [cone and plate(let) analyzer (CPA)] technology under shear stress. The percentage of the well surface coverage (SC) by platelets is calculated. There is significant inverse correlation between the values of SC and the ADAMTS-13 activity, indicating the role of ADAMTS-13 in controlling the extent of platelet adhesion under flow conditions. Based upon generation of fluid shear stress when the reaction mixture is vortexed. Purified plasma VWF substrate is mixed with test plasma and the mixture is subjected to shear stress using a vortex based mixer (for 60 min at 2,500 rpm), mimicking physiological conditions. This generates cleavage products that can be measured using 2.5 % agarose gel and Western Blotting.	[66]
ELISA	Based upon the use of high affinity monoclonal antibodies or polyclonal rabbit anti-human ADAMTS13 IgG antibodies to ADAMTS13, Normal range of protein measured is 951±261 ng/mL per the monoclonal antibody or 740–1,420 ng/mL using the polyclonal antibodies. This method is sensitive and reproducible and is not influenced by the presence of autoantibodies. Potential use in ADAMTS13 antigen determination in diagnosis and follow up in patients with TTP	[67]
SDS-PAGE and Western Blot	A quantitative assay based upon the use of a monoclonal-ADAMTS13 antibody against the TSP1-4 domain. Normal range of ADAMTS13 antigen in 60 healthy subjects was 101.6±49.4 %. Sample plasma is treated with buffer followed by protein separation using SDS-PAGE. After transfer onto a membrane, the blots are probed with a primary monoclonal antibody against ADAMTS13, followed by staining with HRP-conjugated antibody.	[68]

It is important to note that some methodologies of ADAMTS13 measurement are fraught with limitations, the two most important being the use of denaturing agents to unfurl purified VWF and the inability to mimic physiologic high shear stress conditions in vitro. The newer assays use recombinant fragments of VWF rather

Table 3 Physiologic and pathologic conditions in which ADAMTS13 antigen and/or activity levels were examined

Condition	References
Decreased[a]	
Physiologic	
Neonates[a]	[41, 42]
Pregnancy from second trimester and post partum[a]	[41, 42, 44]
>65 years	[41, 42]
Pathologic	
Overweight women	[44]
Liver cirrhosis	[41, 42, 69]
Chronic kidney disease with or without hemodialysis treatment	[42, 70]
Autoimmune diseases including IBD, SLE, SS	[41, 71]
Post operative period of abdominal surgery, Cardiac surgery[a]	[41, 42, 49]
Acute inflammatory state (respiratory bacterial infections, Dengue fever, sepsis)	[42, 51, 72]
DIC	[51]
HIT type 2 and other hematological disorders involving thrombocytopenia	[73]
Increased	
Smokers during pregnancy	[41]
Venous thromboembolism	[74]

IBD inflammatory bowel disease, *SLE* Systemic lupus erythematosus, *SS* systemic sclerosis, *HIT* heparin induced thrombocytopenia

[a]A discrepancy might exist between antigen levels and activity levels

than purified VWF. The fragments harbor the recombinant A2 domain containing the ADAMTS13 cleavage site (e.g. VWF73). Some methods use chemically modified fragments. By using synthetic peptides, substrate specificity has been ensured and the assay is standardized as compared to using purified human VWF with a wide range of VWF multimers. It has been shown that methods based upon modified VWF peptides are a more accurate measurement of ADAMTS13 [40]. It is pertinent to mention that currently there are no international standards, quality control, or officially recognized units of ADAMTS13 activity. The level of ADAMTS13 may be measured by antigen assays which might offer an alternative to activity assays with acceptable coefficients of variation and a smaller standard deviation [41].

6.1 ADAMTS13 Levels in Normal Physiology

Activity levels of plasma ADAMTS13 vary widely amongst healthy individuals and are assay dependent. In general, plasmatic ADAMTS13 activity of 40–170 % is considered normal for healthy individuals [42].

ADAMTS13 activity and antigen levels were examined during various stages of life as described below. The distinct physiologic changes as well as some pathologic changes in ADAMTS13 levels are summarized in Table 3.

6.1.1 Neonates

In two published reports ADAMTS13 activity and antigen levels were lower in neonates as compared to adults [41, 42]. On the other hand Schmugge et al. found normal ADAMTS13 activity levels in the cord blood of most neonates, while 26 % had significantly reduced activity levels [43]. When analyzing activity to antigen levels the ratio was very low compared to normal controls, suggesting that a substantial amount of protease moieties are not active in vitro [41]. Activity levels reached adult levels within a few days after birth to 6 months [42, 43]. The mechanism of this change is not fully understood although immaturity of the newborn liver was suggested [42].

6.1.2 Pregnancy and Post partum

In the first trimester of pregnancy, ADAMTS13 activity levels are similar to levels measured in non-pregnant women. Levels then decrease progressively from the beginning of the second trimester (12–16 weeks of gestation) until the early post partum period (1–3 days after delivery) before beginning to rise again until reaching normal or slightly higher than normal activity levels [42, 44]. Activity levels were found to be lower in nulliparous as compared to parous women and in primigravidas as compared to multigravida women [44]. During pregnancy, activity levels were significantly higher in smokers than in non-smokers [44]. Possible mechanisms to explain the progressive decrease in ADAMTS13 activity include: (1) estrogen regulation in the presence of high levels of estrogens, albeit moderately [42] or (2) higher VWF levels during gestation which consume the protease resulting in lower activity levels [45]. Mannucci et al. speculated that this is a compensating phenomenon meant to dispose of the more platelet adhesive forms of VWF [42]

6.1.3 Elderly

ADAMTS13 activity and antigen levels are lower in healthy individuals aged 65 years or older than in younger individuals [41]. No difference is demonstrated when the ratio of ADAMTS13 activity to antigen is calculated (specific activity) suggesting that although levels are lower the protease is fully enzymatically active.

6.1.4 ADAMTS13 and Thrombotic Microangiopathies (TMA)

TMA is a clinicopathological diagnosis that refers to a combination of microangiopathic hemolytic anemia (MHA), thrombocytopenia, and microvascular thrombosis, regardless of cause or specific tissue involvement [46]. The classification of TMAs has evolved and expanded considerably over the last few decades and correct nomenclature presents a challenge. The discovery of the *ADAMTS13* gene shed light on the pathophysiology of congenital TTP and allowed some distinction from the other causes of TMA.

Differential diagnosis of TMA includes many entities such as cTTP, acTTP, typical and atypical hemolytic uraemic syndrome (HUS), connective tissue diseases such as systemic lupus erythematosus (SLE) and antiphospholipid antibody (APLA) syndrome, as well as certain malignancies and drugs. Plasma ADAMTS13 levels in TMA can vary from normal to undetectable in the various entities with considerable overlap; however, it is important to note that undetectable plasma ADAMTS13 activity is only rarely found in pathological conditions other than TTP and never in any physiologic condition [47–50].

6.1.5 ADAMTS13 Levels in Other Disease States

ADAMTS13 activity and/or antigen levels have been studied in a myriad of pathologic conditions, most of which are known to be associated with increased thrombotic tendency but does not necessarily demonstrate the classic picture of TMA (Table 3). However, the observation of lowered activity and/or antigen level in pathologic conditions does not correlate well with clinically overt thrombotic events [41] nor do we know how accurately a low ADAMTS13 value measured in vitro reflects decreased activity in patients' plasma [51]. Nonetheless, others found that individuals who suffered an early acute arteria thrombotic event, such as stroke, acute myocardial infarction, or peripheral arterial disease were more likely to have lower ADAMTS13 levels as compared to healthy people [52].

Again, ADAMTS13 activity levels in these pathologic conditions, range from undetectable to completely normal. While a clear association exists between undetectable levels of ADAMTS13 activity and the pathophysiologic mechanism behind TTP, such a distinct association does not exist when examining correlations between low levels of ADAMTS13 and other disease states. Whether the low activity levels contribute to the pathophysiology or are the consequence of it remains to be understood.

The interpretation of decreased ADAMTS13 levels in some cases is controversial because ADAMTS13 levels are quite stable in normal plasma, but may deteriorate quickly in plasma samples drawn in pathological conditions hence giving falsely low results [51]. As such, proper handling of samples is of high importance. Another point for consideration when interpreting results involves the inverse relationship between protease activity and VWF levels. Mannucci et al. found that the most important correlate of protease level was the plasma level of its natural substrate VWF [42]. The inverse relationship was further reported in healthy centenarians [53] and during gestation [45], which probably reflects to some extent consumption of ADAMTS13 by higher substrate levels [44].

6.1.6 TTP: The Hallmark of Severely Deficient ADAMTS13

TTP is a life threatening disease with mortality rates exceeding 90 % if left untreated [47]. As mentioned previously, Levy et al. identified that recessively inherited mutations in the *ADAMTS13* gene, either in a homozygous or a compound heterozygous manner, were responsible for congenital TTP (cTTP) [8]. A severe functional

deficiency of ADAMTS13 in the plasma (<5 % of the activity of a normal pooled plasma), most often related to IgG antibody formation, also termed inhibitors [54], is now known as acquired TTP (acTTP). It is by far more prevalent than the congenital form, accounting for more than 90 % of all TTP cases [47]. According to the Orphanet Report Series – Prevalence of rare diseases published in 2012 (Orpha number 54057) http://www.orpha.net/orphacom/cahiers/docs/GB/Prevalence_of_rare_diseases_by_alphabetical_list.pdf, the estimated prevalence of congenital TTP is 25.5/100,000 people. TTP, both congenital and acquired, was traditionally described through a pentad of signs including thrombocytopenia, hemolytic anemia, neurological symptoms, renal impairment and fever, however the classic pentad is now rarely seen and not required for diagnosis. The current definition and diagnostic criteria are microangiopathic hemolytic anemia and thrombocytopenia with or without renal failure or neurological findings with other causes of TMA excluded. ADAMTS13 levels are yet to become part of the diagnostic criteria as in some patients diagnosed with TTP, ADAMTS13 levels are detectable while in others with severe protease deficiency, an alternative cause can be found.

TTP remains a clinical diagnosis and prompt initiation of therapy is crucial in order to decrease mortality rates of untreated patients. Therapeutic plasma exchange (TPE) forms the cornerstone of management. It has been hypothesized that plasma exchange either replenishes the plasma ADAMTS13 or removes inhibitory antibodies. Since plasma exchange has treatment related complications such as hypotension, anaphylaxis, catheter related infection, and thrombosis [55], rapid recognition of patients with suspected TTP who may benefit from it is essential in successful management. TPE will be of no benefit and should be avoided in patients with other "TTP mimetics" (e.g. HELLP, infections, malignant hypertension etc.) because of the risks involved. Response to therapy is variable with some patients responding quickly and completely and others having a protracted course with exacerbations or failure to respond. It has been noted that patients with high titer inhibitory antibodies have a higher chance of treatment failure. Treatment with steroids and immune modulator drugs like rituximab and cyclosporine aid in the management of exacerbations, recurrence, or relapsed disease [56]. In the congenital form of TTP clinical course is highly variable. Almost half of the patients will manifest as neonates while others will present symptoms only in childhood or as adults. In some patients, episodes are triggered by infections or medications and women might present only when pregnant [57]. Disease manifestations change considerably between patients and even between siblings who carry the same mutation. The corners of treatment is fresh frozen plasma infusions to provide the deficient protease as autoantibodies are rarely seen in cTTP. These infusions can be administered at the onset of each episode; prophylactic treatments are employed in patients with chronically relapsing course.

7 Conclusions

The discovery of the von Willebrand Factor cleaving protease and its implication in a myriad of thrombotic microangiopathies and inflammatory states has been a significant advancement in medicine over the last 50 years. Much has been added to

our knowledge of this unique protease. Significant gains have been made in understanding the molecular mechanisms of TTP and the genetics of *ADAMTS13* in health and disease while knowledge is evolving in other areas. Understanding the correlations between phenotypic expression and ADAMTS13 mutations and the impact of synonymous variations is on the current forefront of research. Other frontiers to be conquered are refining the molecular interactions of VWF and ADAMTS13 and using ADAMTS13 as a prognostic tool and potential target for therapy in inflammatory states and stroke. New treatment modalities for TTP are needed as mortality rates continue to be high in the era of modern medicine and advanced supportive care. Recombinant ADAMTS13 is under development and will hopefully enrich current armamentarium. In a KO *ADAMTS13* murine model, recombinant ADAMTS13 was shown to protect the mice from developing TTP [58]. Further research is needed to refine these modalities and for their incorporation into clinical medicine

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Role of Proteases and Their Tissue Inhibitors in Pregnancy Outcome

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Abstract Mammalian pregnancy is the successful outcome of series of events comprising of effective fertilization of male and female gamete, controlled growth and development of the embryo in the oviduct, transport to the uterus, attachment and implantation in the endometrium. These processes are coordinately and principally regulated by specific steroid hormones, cytokines and growth factors. Recently, matrix metalloproteinases (MMPs) and their tissue inhibitors of metalloproteinases (TIMPs) have been found to coordinate these events in major ways. They have been found to be involved in folliculogenesis, ovulation, corpus luteum turnover in ovary and degradation and regeneration uterine endometrium (known as endometrial tissue remodeling) during estrous cycle, implantation and placentation that are required for successful pregnancy outcome. The activities of these proteases and their inhibitors change dynamically to release healthy oocytes from ovary and during early pregnancy to render the uterine microenvironment suitable for attachment and implantation of growing embryo and to transform it in to a developed fetus through establishment of feto-maternal circulation. There exists a fine balance between the activities of these proteases and their inhibitors to regulate these events. Any breach in the equilibrium of the proteases and their inhibitors in the ovary or uterine microenvironment or in their regulation may pre-dispose a condition of failed pregnancy. An account of such proteases and their inhibitors and their role in pregnancy outcome has been discussed in this chapter.

Keywords Extracellular matrix • Implantation • Matrix metalloproteinases • Pregnancy • Recurrent pregnancy loss • Tissue inhibitors of metalloproteinases • Tissue remodeling

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1 Introduction

Mammalian ovary and uterine endometrium are specialized tissues that continuously undergo dynamic structural and physiological changes according to the stage of the estrous cycle and pregnancy. The structural changes of the ovary includes growth and expansion of follicles through the process of folliculogenesis, ovulation, formation and regression of corpus luteum (CL) and that of endometrium includes an extensive but controlled degradation and regeneration of the extracellular matrix (ECM) known as 'ECM/tissue remodeling' to provide a suitable microenvironment for embryonic development, trophoblast invasion, implantation of conceptus (embryo and associated membranes) and placentation leading to establishment of pregnancy. These dynamic changes in ovary and endometrial architecture are orchestrated by a network of steroid hormones that includes progesterone and estrogen, an array of metal-dependent proteases and their inhibitors known as matrix metalloproteinases (MMPs) and tissue inhibitors metalloproteinases (TIMPs), growth factors and cytokines [1, 2]. In recent years, this MMP-TIMP system of ovary and ovary and endometrium has taken a centre stage for understanding the complicacies of polycystic ovarian syndrome (PCOS), endometriosis, pre-eclampsia and other conditions leading to failed pregnancy.

2 The MMP-TIMP System

The MMPs family, also called matrixins, encompasses at least 23 related proteolytic enzymes and has the ability to degrade most, if not all, components of the ECM through a zinc-catalyzed mechanism and regulate cell migration, proliferation, differentiation and invasion [2, 3]. Based on the structure and substrate specificity, MMPs have been classified broadly into six categories; viz., (1) the collagenases (MMP-1, -8, -13); (2) the gelatinases (MMP-2, -9); (3) the matrilysins (MMP-7, -26) (4) the membrane type (MT-MMP-14, -15, -16, -17, -24, -25); (5) the stromelysins (MMP-3, -10, -11, -12) and (6) other MMPs (MMP-19, -20, -21, -23, -27, -28) [4]. All MMPs share common structural features; consisting of a (1) prepeptide (20 residues, that target MMPs to the endoplasmic reticulum), (2) prodomain (about 80 residues, confers the latency form of the enzyme), (3) catalytic domain (160–170 residues, having a Zn^{2+} binding site for its catalytic activity) (4) a hinge region (except for matrilysins) and (5) hemopexin (about 200 residues, involved in substrate recognition) [4]. MMPs are synthesized and secreted as inactive zymogens (proforms) and are activated proteolytically and sequentially either during or after secretion into the extracellular space by various enzymes (including MMPs) and cytokines and in some cases remain membrane bound [5, 6]. The expression and activities of most MMPs and TIMPs are transcriptionally regulated by hormones, growth factors, cytokines and micro RNAs in a temporal and spatial manner [4, 7]. The catalytic activities of these MMPs are inhibited by serum-born

(e.g., α2 macroglobulins) and tissue-derived inhibitors (e.g., TIMPs) [8, 9]. TIMPs are the major endogenous inhibitors of MMP activities and four homologues (TIMP-1, -2, -3 & -4) have been identified so far [3]. TIMPs have six disulfide bonds and consist of an N-terminal domain that inhibits MMP activity by occupying the catalytic cleft, and a C-terminal domain that is able to bind to the hemopexin domain of the gelatinases [10]. They are locally produced and hormonally regulated and proposed to coordinate various ovarian and uterine processes. They have also been shown to inhibit migratory and invasive activity of cells, tumorigenesis, metastasis and angiogenesis [11].

Extracellular matrix degradation is a precisely controlled process and homeostasis of this process is maintained by activation and deactivation of MMP-TIMP system. The signal peptide i.e. the pre-pro peptide is removed during translation that needs to be activated further by removal of the pro-peptide masking the catalytic site of the enzyme. The cleavage of pre-peptide of MMPs starts with action of plasma proteases such as plasmin or kallikreins which is followed by complete removal of pro-peptide by another MMP [11]. MMPs are entrapped within the four identical subunits of α_2-macroglobulin and the complex is then rapidly cleared by receptor-mediated endocytosis via the low density lipoprotein receptor-related protein-1 (LRP-1) [12]. There exists a delicate balance between the activities of MMPs and TIMPs for a controlled regulation of ECM remodeling. Any breach in these balance of MMP-TIMP system leads to various disease conditions including reproductive disorders in vertebrates.

3 Role of MMP-TIMP System in Follicullogenesis, Ovulation, and CL Formation

Release of a healthy oocyte from the ovary is an absolute requirement for successful fertilization. The growth and development of the ovarian follicles are controlled by steroid hormones. There is extensive cellular proliferation and remodeling of the ECM as the follicle grows and differentiates along the path from a small primordial follicle to a large preovulatory Graafian follicle leading to ovulation and all these processes are coordinately regulated by the MMP-TIMP system. In rodents, the expression of mRNA for MMP-2 and MMP-9 has been localized to the theca of developing follicles and to the ovarian stroma [13]. The mRNAs of TIMP-1, -2 and -3 were localized to the stroma and theca of developing follicles. Similarly, TIMP-3 has been localized in the granulose cells [14, 15]. Further, in rodents MMP-2, -9, -13 and TIMP-1 and -4 were reported to be induced in ovary during folliculogenesis [16]. In goat, MMP-2 and -9 activities have been noted to increase with the increase in follicular size [17]. The secretion of MMPs has been correlated with the quality of the developing follicle and it was suggested that MMPs can be used as markers for assessment of growth and development of ovarian follicles [18]. In sheep, diversion of normal follicles to atretic follicles was associated with an increased

intrafollicular levels of MMP-2 and -9 activities [19]. During the process of ovulation, MMP-1 protein was immunolocalized in theca interna and externa, interstitial glands, and germinal epithelium. MMP-19 and TIMP-1 mRNA were found in theca interstitial cells. The mRNA expression of MMP-1, -10, and MMP-19 were up regulated during ovulation in rhesus macaque ovulatory follicle and were found to be critical for follicle rupture in primates [20].

Formation of the CL was associated with an increased gelatinolytic activity. An administration of hCG induced the expression of TIMP-1 and TIMP-3 mRNA in luteinizing granulosa cells. The luteal cells expressed TIMP-1, -2 and -3 mRNA with unique pattern of cellular expression for each of the TIMPs in a newly formed CL Regression of the CL was also associated with a significant increase in the activity of the metalloproteinases [2].

In the cycling rat ovary, basigin (Bsg), a putative regulator of MMP induction, was localized to newly forming CL, the theca of preovulatory follicles, and appeared to be lower in CL from previous estrous cycles. Both functional and structural regression of CL was associated with a decline in Bsg expression level. However, treatment of cultured granulosa cells with hCG significantly augmented Bsg mRNA expression. Thus it was proposed that the process of ovulation and luteogenesis may be facilitated by Bsg expression [21].

4 MMP-TIMPs in Estrous or Menstrual Cycle

Menstruation in primates is a natural process wherein there is loss of endometrium tissue and blood vasculature. This irreversible tissue breakdown of endometrium is mediated by MMPs [22]. An increased expression of MMPs including MMP-1, -2, -3, -7, -8, -9 and -12 have been reported in menstruation [2, 23, 24]. The mRNA expression, the corresponding protein synthesis and secretion in to the epithelial lining and release of the secreted MMP protein is highly compartmentalized [25] as stromal cells are source of MMPs and neutrophils that infiltrate the endometrial tissue during menstruation also express MMP-2, -9, -14, and -15 [26–28] and MMP-7 is epithelial cell specific [29] and some MMPs have focal expression pattern [30, 31]. The expression of MMPs in endometrium is primarily regulated by ovarian steroids as the withdrawal of progesterone in tissue culture models have led to significant increase in MMP-1, -2, -3 and -9 in endometrial stromal cells without any change in TIMP-1, -2 and -3 levels [32–35]. Cultured ovine and bovine endometrial cells have been shown to secrete MMPs (viz., MMP-2 and -9) and TIMPs (viz., TIMP-1, -2, and -3) in the medium [36–39]. The MMP-2 proteins and transcripts are up regulated both in endometrial stromal and epithelial cells during menstruation but also present in all the phases of menstrual cycle in humans whereas MMP-9 is expressed only during menstruation in the same species, which primarily reflects a stage-specific expression in response to cyclic changes in circulating steroid hormone levels [1, 23]. Thus, the balance of MMP-TIMP system is shifted more towards MMP activation at menstruation for an irreversible tissue breakdown.

The activation, inactivation, clearance and regulation of MMP-TIMP system during menstruation has been reviewed recently by Gaide Chevronnay et al. [4].

5 Role of MMP-TIMP System in Implantation and Placentation

During implantation window or endometrial receptivity, the endometrium undergoes a series of structural changes so as to render the endometrium ready to receive the incoming blastocyst for implantation. During this period a precise cross-talk happens between the invasive blastocyst and receptive endometrium. Before implantation, the blastocyst also prepare itself for trophoblast differentiation with invasive properties. If, for any reason, this cross-talk is perturbed, then it leads to various pregnancy-related disorders leading to embryonic loss. The implantation process involves a variety of molecules that includes proteases and their inhibitors, steroid hormones, growth factors, cytokines and a variety of cell adhesion molecules. Recently, studies pertaining to the roles of MMPs and TIMPs in implantation and placentation during early pregnancy have taken a centre stage due to their importance in various pregnancy-related disorders. Invasion of trophoblast cells in to the endometrial epithelial and stroma layers is controlled by balanced activities of MMP-TIMPs system. In human, the implantation during weeks 3–9 of pregnancy was associated with an increase in mRNA expression and the corresponding activities of MMP-9, TIMP-1 and -3 in extravillous cytotrophoblast cells and that of TIMP-2 decreased at weeks 3–9. A functional network of vascular endothelial growth factor, insulin growth factor 2, and MMP-9 in early placental trophoblast cells and maternal endometrium was reported to be important for normal placentation [40]. During early pregnancy, the expression of MMPs have been implicated in the invasion of endometrium by trophoblast cells during implantation in human [41], mouse [42] and bovine [39, 43, 44]. In non-primate species such as buffalo also, this estrous cycle and pregnancy-dependent modulation of MMPs is required for dynamic remodeling of the endometrium during such processes [45].

In human and mice, trophoblasts are highly invasive and penetrate into the endometrial stromal tissues [46]. Whereas in ruminants, the placenta is of synepitheliochorial type and trophoblasts are less-invasive since the binucleate trophoblast cells migrate and fuse with the luminal epithelial cells without crossing the basal lamina of the endometrium to form multinucleated syncytium [47]. Thus, the endometrial tissue remodeling and the underlying mechanism during estrous cycle and early pregnancy in ruminants may be different than that of humans and most primates. MMP-1, -2 and TIMP-2 proteins have been demonstrated in pregnant bovine endometrium and placentome [43, 44]. In bovine, differential expressions of MMP-2 and -9 transcripts were demonstrated in endometrium and placentome throughout gestation [48] and a lower TIMP-2 protein level was demonstrated in day 16–18 pregnant ULF compared to non-pregnant [49]. Recently, high concentrations of MMP-9 in serum was positively correlated with successful in vitro fertilization outcome (pregnancy) in females [50].

Table 1 Status of MMPs and TIMPs in various pregnancy-related disorders of human

Name of the disorder	Status of specific MMP	Status of specific TIMP	References
Polycystic ovarian syndrome (PCOS)	Granulosa cells: MMP-2, -9↑ Follicular fluid: MMP-2, -9↑	Follicular fluid: TIMP-1, -2 ↑	[60]
Endometriosis	–	Peritoneal fluid: TIMP-1↑	[61]
Preeclampsia	Placental: MMP-9↑	–	[32]
	Endothelial cells: MMP-1↓	–	[64]
	Placenta: MMP-10, -13 and -15 mRNA↑	Placenta: TIMP-2, -3 mRNA↑	[65]
Anovulatory dysfunctional uterine bleeding (ADUB)/ endometrial hyperplasia	Endometrium: MMP-2, -9↑	–	[63]
Idiopathic recurrent spontaneous abortion (IRSA)	Endometrium: Single nucleotide polymorphism in MMP-2 and MMP-9 genes	–	[56]
	Serum: MMP-2, -9↑	–	[57]
Recurrent pregnancy loss (RPL)	Serum: MMP-3↑	Serum: TIMP-3↑	[55]
	Endometrium: MMP-2 mRNA↑	Endometrium: TIMP-3 mRNA↓	[51]
	Uterine luminal fluid: MMP-9↓	Uterine luminal fluid: TIMP-1↓	[53]
Pre-term labour	Serum: MMP-9↑	Serum: TIMP-1↓ Serum: TIMP-2↓	[66]

Note: ↑: increased; ↓: decreased

6 Imbalance of MMP-TIMP System, Utero-Ovarian Disorders and Failed Pregnancy

An imbalance in MMP-TIMP system in humans has been implicated for various reproductive disorders including PCOS, follicular atresia, endometriosis, preeclampsia, anovulatory dysfunctional uterine bleeding, idiopathic recurrent spontaneous abortion, recurrent pregnancy loss, preterm labor etc. [51–57]. Table 1 describes the status of MMPs and TIMPs in various pregnancy-related disorders of human.

In luteinized granulosa cells from women with polycystic ovarian syndrome (PCOS), the MMP-TIMP balance is shifted toward greater MMP activity [58, 59]. Significantly higher levels of MMP-2 and MMP-9 were secreted from cultured luteinized granulosa cells obtained from PCOS patients compared to granulosa cells from normal ovulatory patients whereas the secreted basal level of TIMP-1 was similar in both types granulosa cells. Thus these results points to a higher net gelatinolytic activity with the luteinizing granulosa cells of patients with PCOS. Similarly, women with PCOS exhibited significantly increased MMP-2, MMP-9, TIMP-1 and TIMP-2 activities in follicular fluid compared with controls of similar age [60]. In sheep, diversion of normal follicles to atresia by hypophysectomy was followed by

a significant increase of intrafollicular levels of MMP-2 and MMP-9. Thus, MMP-2 and MMP-9 may be associated with inappropriate PCOS. Similarly, in a rat model of endometriosis, excessive TIMP1 was secreted into the periotoneal fluid and it was deleterious to ovulation and embryo development as the balance of MMP-TIMP system was altered [61] and recently, the action of TIMP-1 was found to be both dependent and independent of MMP inhibition [62].

In human, during the period of maximal endometrial receptivity i.e. implantation window (7–9 days after ovulation), the endometrium prepares itself for implantation of developing embryo. Any dysregulation of MMP-2, MMP-9, and TIMP1 expressions are associated with infertility and early pregnancy loss [53]. Endometrial hyperplasia of women with anovulatory dysfunctional uterine bleeding was associated with enhanced expressions of MMP-2 and -9 [63]. Idiopathic recurrent spontaneous abortion (IRSA) in females has been associated with abnormalities in the remodeling of endometrial extracellular matrix as well as aberrant MMP expression in endometrium [57]. Single nucleotide polymorphism in MMP-2-735 C/T and MMP-9-1562 C/T functional genes increased the risk of IRSA in women [56]. Recently spontaneous early pregnancy failure was associated with an increased serum concentration of MMP-2 and -9 [57]. In preeclampsia, there was reduced expression of MMP-1 but increased expression of MMP-10, -13 and -15 by the cytotrophoblasts and it leads to failure of trophoblasts to invade maternal decidual blood vessels [64, 65]. In women, an imbalance between MMPs and TIMPs have been implicated in the pathogenesis of preterm labor as the serum concentration of MMP-9:TIMP-1 and MMP-9:TIMP-2 were found to be tilted in favour of gelatinolysis during preterm labor [66].

In recurrent pregnancy loss of women, the endometrium tissue was associated with an increased mRNA expression of MMP-2 and decreased expression of TIMP-3 [51]. However, serum concentrations of MMP-3 and TIMP-3 were increased [55]. The uterine luminal fluid demonstrated a decreased activity of MMP-9 and TIMP-1 [53].

7 Conclusions

Thus, depending upon the stage of the estrous or menstrual cycle and pregnancy, the MMP-TIMP system plays a critical role for ovarian follicular turnover, corpus luteum formation and regression and endometrial tissue remodelling. The steroid hormones, cytokines, growth factors, cellular receptors and micro RNAs form a interacting network to regulate the activities of these proteases and their inhibitors for a controlled remodelling of the utero-ovarian extracellular matrix endometrium so as to provide a suitable microenvironment for effective fertilization, growth and development of embryo and establishment and maintenance of pregnancy. The molecular nature of these proteases and their inhibitors and the extent of involvement of a specific protease may differ according to the species and the type of placentation. Any breach in the regulation of MMP-TIMP system by any external or internal stimuli may

predispose the subject to a failed pregnancy outcome. The future strategies should include identification of factors that may balance the MMP-TIMP system and any other agent that may neutralize the effect of excessive proteases or inhibitors that are responsible for MMP-TIMP related utero-ovarian pathologies.

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Matrix Metalloproteinases in Bone Health and Disease

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Abstract During bone development the extracellular matrix (ECM) undergoes extensive modeling and remodeling by different proteases including members of the matrix metalloproteinase (MMP) family. The most dominant MMPs in bone development are the gelatinases MMP-2 and MMP-9, the collagenase MMP-13 and the membrane-bound MT1-MMP. The enzymes are secreted by different cells in the bone microenvironment, including osteocytes, osteoblasts, osteoclasts, chondrocytes, and endothelial cells. In endochondral bone development, MMPs are involved as early as the initial vascularization of the cartilage anlage while later they regulate chondrocytes proliferation, differentiation, and apoptosis at the growth-plate, as well as vascularization at the chondro-osseous junction. At sites of bone resorption the relative importance of MMPs for matrix degradation depends on the bone type: they participate in resorption of calvarial but not long bones while in the latter they are significant for osteoclasts migration and invasion. The importance of MMPs in bone development is emphasized by several bone-related syndromes in human with single mutations in MMP genes. These, together with targeted mutation in animal models shed light on the role of different MMPs in many aspects of bone development. In this view it is not surprising that MMPs also participate in pathological conditions in bones. They play significant role in migration and establishment of tumor metastasis into bone and tumor-induced osteolysis; they are dominant in the degradation of collagen type I during the course of osteoarthritis; and are even involved in fracture repair. In this chapter we summarize the current knowledge regarding the central role of MMPs in bone health and disease.

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1 Introduction

This chapter summarizes the current knowledge regarding the role of MMPs in bone health and disease. These enzymes take part in bone resorption, allowing bone modeling and remodeling and in endochondral ossification, allowing bone elongation. Their importance in bone biology is especially notable in human bone-related syndromes caused by mutations in MMP genes. Their function in different aspects of bone development was studied using targeted mutations in animals as well as studies with MMP inhibitors, and experiments in both *in-vivo* and *in-vitro* systems.

Two distinct processes lead to bone development: the flat bones of the skull develop by intramembranous ossification in which mesenchymal cells differentiate to osteoblasts, whereas the long bones and vertebrae are formed by endochondral ossification in which chondrocytes first proliferate and differentiate to form a zone of hypertrophic cartilage that is then resorbed and replaced by mineralized bone matrix. The resorption of the extra cellular matrix (ECM) of the hypertrophic cartilage by osteoclasts makes a way for invading capillary endothelial cells, enabling osteoblast's recruitment and formation of the trabecular bone. After birth, this process continues at the growth plates located at the end of the long bones [1]. Throughout this process a non-vascularized tissue (cartilage) is converted into one of the most vascularized tissues in the body (bone) with complete remodeling of the ECM. In addition, remodeling of existing bone into newer bone takes place throughout life and ensures mineral homeostasis and optimal mechanical properties of the skeleton. This is accomplished through the coordinated action of bone-resorbing osteoclasts and bone-forming osteoblasts [2]. Taken together, it is clear that remodeling of ECM components is critical for development, growth, and homeostasis of the bone, making proteinases highly significant for bone development.

Several Matrix metalloproteinases (MMPs) are involved in bone development. The MMPs belong to a family of zinc-dependent proteolytic enzymes that degrade numerous pericellular substrates, including components of the ECM, other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor–binding proteins, cell surface receptors, and cell-cell adhesion molecules [3]. Due to their broad actions, MMPs are not merely extracellular proteases; they influence cell proliferation, differentiation, motility, remodeling, wound healing, angiogenesis, and apoptosis [4]. To date, 28 family members were identified, calcified in six main subfamilies: collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MT-MMPs: MMP-14, -15, -16, -17, -24 and -25), and other MMPs, which are not categorized in

any of the previous groups (MMP-12, -19, -20, -21, -23, -27 and -28) [5]. MMP-2, 9, 13, and MT1-MMP were shown to be critical for the key events of bone growth such as marrow cavity formation, capillary invasion, matrix remodeling and eventually replacement of cartilage by bone.

2 MMPs in Normal Bone Maintenance

Main contributions of different MMPs in bone development are summarized in Table 2.

2.1 MMPs in Endochondral Ossification

Several MMPs are highly expressed during endochondral ossification. MT1-MMP and MMP-9 are expressed by osteoclast at early stages, when the vascularization of the cartilage anlage begins, and MMP-13 is expressed by terminal hypertrophic chondrocytes and osteoblasts. MMPs are involved in proliferation, differentiation, and apoptosis of growth plate chondrocytes through the modulation of growth factors, cytokines, adhesion receptors, and other enzymes. In the human growth plate several MMPs were identified: MT1-MMP was the most prominent MMP within all zones of the growth plate. MMP-1, 9, 10, 11, and 13 were expressed in hypertrophic chondrocytes and osteoblasts. MMP-2 was expressed in the reserve zone and hypertrophic chondrocytes MMP-2 and 9 were also detected in osteoclasts [6]. The role of these MMPs in endochondral ossification is understood mainly through human mutations and usage of null mice. The major results of these studies are summarized here.

2.2 MMPs Contribution to Osteoclasts Activity

Osteoclasts are multinucleated cells responsible for bone resorption, by activating efficient machinery for dissolving crystalline hydroxyapatite and degrading organic bone matrix rich in collagen fibers. When initiating bone resorption, osteoclasts become polarized, and three distinct membrane domains appear: a ruffled border, a sealing zone and a functional secretory domain. Extensive vesicle transport to the ruffled border delivers hydrochloric acid and proteinases to an area between the ruffled border and the bone surface called the resorption lacuna. In this extracellular compartment, crystalline hydroxyapatite is dissolved by acid, and a mixture of proteinases degrades the organic matrix [7]. The proteinases which were found as rate-limiting for osteoclastic bone resorption are a group of cysteine proteinases, in particular cathepsin K, and MMPs [8–10]. The relative importance of cysteine proteinases and MMPs depend on the bone type: resorption of calvarial bones depends

on the activity of cysteine proteinases and MMPs, whereas resorption of long bones depends primarily on the activity of cysteine proteinases [11]. In addition, the two families act in series rather than in parallel at different stages of the resorption cycle: first, osteoclast attachment to the mineralized surface creates an area with a low pH in which the mineral crystallites are dissolved. Then cysteine proteinases (active at such a low Ph) digest part of the bone matrix, and finally, when the pH has increased, MMPs exert their activity [12].

The access of osteoclasts to the bone surface is an early determinant of bone resorption and has important implications for bone morphogenesis, repair, and maintenance. Osteoclastic MMPs facilitate osteoclast migration through collagen and are critical for the access of the osteoclast to its future resorption site [13]. Specifically, MMP-9 is essential for osteoclast migration and invasion into the hypertrophic cartilaginous core of the bone rudiments as part of the marrow cavity formation. MMP-9 is not involved in the removal of mineralized matrix but rather regulates proteolysis of non-mineralized cartilage, leading to the release of ECM-bound vascular endothelial growth factor (VEGF), which is a chemo-attractant for osteoclasts and blood vessels [14]. At later stages, MMP-9 is expressed at the chondro-osseous junction by endothelial cells and osteoclasts. It attacks the edge of the endochondral cartilage and helps to solubilize the type II-collagen-rich fibrillar framework, which is then released as debris for further digestion, probably by MMP-13. This final step opens the way to invasion by capillaries, thereby making possible the replacement of cartilage by bone [15].

MT1-MMP, a membrane-bound collagenase, is also involved in the invasive activity of osteoclasts and their anchoring to the bone surface. MT1-MMP has been identified in the lamellipodia and on podosomes of osteoclasts. These structures are related to the migration and attachment (respectively) of the osteoclasts to the bone surface, thus the cytoskeletal elements bring MT1-MMP into contact with the specific sites of the extracellular matrix that must be altered to allow cell movement or attachment [16]. Besides its ability to degrade different extracellular matrix proteins, MT1-MMP is also able to activate extracellular proMMP-2 [17] and interstitial proMMP-13 [18] which in turn may degrade various extracellular matrix proteins.

2.3 Non-Osteoclastic MMPs in Bone Matrix Solubilization

Several osteoclastic MMPs able to solubilize collagen have been identified in bone areas undergoing resorption. They include interstitial collagenases such as MMP-13 and MT1-MMP, the gelatinases MMP-2 and MMP-9, and other MMPs like MMP-12. However, none of these MMPs were found to be critical for bone resorption in either long bones or calvariae. In contrast MMP-13, originating from peri-osteoclastic osteocytes was shown to participate in resorption. It is translocated from the osteocytes into the resorption site and found at the bottom of the resorption lacunae (reviewed by Delaisse et al. 2003) [19].

After withdrawal of osteoclasts from the resorption pit, bone lining cells enter the lacuna and secrete MMPs which remove demineralized collagen that was not digested by the osteoclasts. This "cleaning" is prerequisite for the subsequent deposition of a first layer of collagenous proteins in the Howship's lacuna, in close association with an osteopontin-rich cement line. This appears to be an obligatory step in the link between bone resorption and formation. Although the type(s) of MMP involved in this process have yet to be determined, possible candidates are MMP-2, MMP-13 or a MT-MMP [20]. It is suggested that the bone lining cells, through small vesicles in cytoplasmic extensions, transduce signals to and from osteoclast. This interaction enables the following mechanism: (1) before osteoclastic attachment, bone lining cells digest nonmineralized collagen protruding from the bone surface. This digestion depends on activity of MMPs. (2) Osteoclasts attach to these sites and resorb bone, but the resorption is incomplete and demineralized nondigested bone is remained in these sites. (3) Bone lining cells enter resorption lacunae and digest the collagen left by osteoclasts in MMPs-dependent activity. (4) Bone lining cells form a cement line and deposit a thin layer of fibrillar collagen on the cleaned surfaces followed by osteoid deposition and actual bone formation by osteoblasts.

MMPs also play a significant role in facilitating osteoclastic bone resorption; it was shown that MMPs, in particular collagenases, are secreted from osteoblasts to destruct the osteoid layer which covers bone surfaces and acts as a barrier between osteoclasts and underlying mineral. This exposes bone mineral to osteoclastic contact and enables the initiation of degradation [21, 22]. Osteoblast produce collagenases in response to bone resorptive substances such as PTH, prostaglandin E2, and 1,25(OH)2 vitamin D-3 [23].

3 Spontaneous Mutations in Human MMP Genes

Mutation in MMP genes were identified as the cause of several bone-related syndromes in human. Mutations in MMP-2 and MT1-MMP are now known as the cause for a range of syndromes characterized by severe osteolysis and mutations in MMP-13 cause defective growth and modeling of vertebrae and long bones. The phenotype of different mutation in MMP genes in human are summarized in Table 1.

3.1 MMP-2 Mutations

In 2000, Al-Mayouf et al. reported on an autosomal recessive disease characterized by simultaneous presentation of multicentric nodulosis, arthropathy and osteolysis (NAO, MONA). Some patients showed deformed and painful hands. All the patients had osteopenia and undertubulation (failure in modeling) of bones. Osteolysis was seen in carpal and tarsal bones. Other common findings were sclerotic cranial

Table 1 Effect of loss in MMP expression in human mutations and mice models

	Phenotype of human mutation	Phenotype of null mouse
MMP-2	Osteolysis	Disruption of the osteocytic networks
	Osteopenia	Reduced bone density
	Failure in bone modeling	Calvarial sclerosis (in old mice)
	Sclerotic cranial sutures	Attenuated osteopenia and joint defects
	Brachycephaly	Articular cartilage destruction and Abnormal long bone and craniofacial development
	Broad medial clavicles	Reduced bone strength
	Facial anomalies	
	Short stature	
MMP-9	—	Shortening of long bones
		Delayed endochondral ossification
		Delayed chondrocyte apoptosis
		Delayed vascularization and ossification
		Defects in osteoclast recruitment
		Decreased bone toughness
MMP-13	Failure in bone growth and modeling	Wide growth plates with longer hypertrophic zone
	Pear-shaped vertebrae	Disordered chondrocyte columns
	Bowing of the femora and/or tibiae	Defects in primary ossification
	Predisposition to osteoarthritis	Impaired vascularization
		Increased trabecular bone
MT1-MMP	Skeletal and joint deformities	Delayed ossification of calvarial bones
	Osteolysis	Incomplete suture closure
	Interphalangeal joint erosions	Cranial dysmorphism
	Osteoporosis	Shortening of long bones
	Joint loss of function	Osteopenia and reduced bone mass
		Generalized arthritis
		Overgrowth of synovial tissue
		Destruction of articular cartilage
		Increased cell proliferation, vascularity, and fibrosis of tendons, ligaments, synovial capsules, musculo-tendinal junctions, and septal/fascial structures associated with skeletal muscle
		Defects in secondary ossification
		Disorganization and lack of chondrocyte proliferation with thickened hypertrophic zone in the growth plate
		Impairment of osteogenic capacity and collagenolytic/gelatinolytic activity of marrow stromal cells

sutures, brachycephaly, and broad medial clavicles [24]. In the same year, Al Aqeel et al. described a case of two sibs with facial anomalies and short stature. The sibs displayed a distal arthropathy of the metacarpal, metatarsal, and interphalangeal joints and resulted in crippling ankylosis and severe generalized osteopenia. Facial changes included proptosis, a narrow nasal bridge, bulbous nose, and micrognathia. In addition, they had large, painful fibrocollagenous palmar and plantar pads and mild body hirsutism [25]. A year later, it was discovered that the origins for these

syndromes are mutations in the gene encoding MMP-2, resulting in no MMP-2 activity in the serum of the patients. Two homoallelic MMP-2 mutations were identified: R101H, a missense mutation that predicted the substitution of an arginine by a histidine which disrupts hydrogen bond formation within the highly conserved prodomain adjacent to the catalytic zinc ion and Y244X, a nonsense mutation predicting the replacement of a tyrosine residue by a stop codon causing a deletion of the substrate-binding and catalytic sites and the fibronectin type II-like and hemopexin/TIMP2 binding domains [26]. In 2009, a novel MMP-2 mutation causing NAO was identified. This frame- shift mutation in amino-acid residue N577 cause deletion of the terminal half of the enzyme's hemopexin domain, a region important in substrate binding and specificity. Serum from the affected individuals was notable for almost complete absence of enzymatic activity. In addition to carpal and tarsal osteolysis, interphalangeal joint erosions, facial dysmorphia, and presence of fibrocollagenous nodules, these patients also possessed congenital heart defects [27]. NAO belongs to a group of three inherited osteolysis syndromes that show autosomal recessive inheritance and multicentric involvement and are also referred to as "the vanishing bone syndromes". The two other syndromes are Torg and Winchester syndromes. In 2005, a mutation in MMP-2 was identified, that causes Winchester syndrome. This missense mutation, E404K, resulted in the substitution of glutamate at position 404, a key amino acid in the catalytic domain of MMP-2 with lysine. The patient showed shortening of the trunk and limbs and marked brachydactyly of the hands and feet as well as severs osteolysis [28]. The involvement of MMP-2 in Winchester syndrome was further augmented by the identification of an in-frame homozygous deletion which resulted in the loss of a valine residue at position 400. This highly conserved amino acid is located in the catalytic domain of the MMP-2 protein [29].

The mechanism explaining how loss of a single proteolytic enzyme results in an apparent increase in bone loss in these syndromes is unclear. One possibility is that the MMP-2 deficiency and the resultant extracellular matrix breakdown defect would create an imbalance between bone synthesis and resorption, thereby resulting in an overall osteolytic phenotype [26]. Another suggested possibility is that the failure of matrix degradation leads to osteocyte and osteoblast apoptosis as was described in mice carrying a targeted mutation in *Col1a1*, encoding a collagenase-resistant form of type I collagen, however those mice also showed increased bone deposition [30]. We suggest that the osteolytic phenotype is not connected only to the deficiency of the basic function of MMP-2 as a proteinase that merely breaks-down the ECM, but rather to its functions in regulating cell-matrix interactions, growth and availability of bioactive molecules.

3.2 MMP-13 Mutation

Missense mutation of MMP-13, in which F56S substitutes an evolutionarily conserved phenylalanine residue for a serine in the pro-region domain of MMP-13 causes the Missouri type of human spondyloepimetaphyseal dysplasia (SEMDMO),

an autosomal dominant disorder characterized by defective growth and modeling of vertebrae and long bones. SEMDMO is characterized by moderate to severe metaphyseal changes, mild epiphyseal involvement, and pear-shaped vertebrae in childhood, with rhizomelic shortening especially of the lower limbs and genu varum deformities secondary to bowing of the femora, tibiae, or both. The modeling defects improve spontaneously by early adolescence, yet affected individuals remain shorter than unaffected siblings. Additionally, bowing deformities predispose to osteoarthritis, especially of the knees [31].

3.3 MT1-MMP Mutation

Winchester syndrome is a autosomal-recessive disorder causing severe skeletal and joint deformities with progressive bilateral and symmetric osteolysis of the carpals and tarsals, interphalangeal joint erosions mimicking rheumatoid arthritis, generalized osteoporosis, and eventual loss of function of the larger joints, including the shoulder, elbow, hip, and knee. The cause for this syndrome is a missense mutation in the gene encoding MT1-MMP that predicted replacement of a threonine by an arginine. The mutation decreases MT1-MMP membrane localization with consequent impairment of pro-MMP-2 activation, thus a reduction in MMP-2 activity is also observed in these patients [32].

4 Animal Models: Targeted Mutations in Mmp Genes

In an attempt to understand the exact role of the different MMPs as well as the mechanisms mediating the bone-related syndromes in human carrying MMP mutations, several models of MMPs knock-out mice were generated. These models provided vast amount of information regarding the role of MMPs in bone and growth plate development. The current data is summarized here. The phenotype of targeted mutation in Mmp genes in mice are summarized in Table 1.

4.1 Mmp-2 Null

The first detailed characterization of bones from *Mmp2*$^{-/-}$ mice was described by Inoue et al. 2006. Surprisingly, the characteristic nodulosis, arthropathy, and focal osteolysis of the human disease were not observed in the *Mmp2*$^{-/-}$ mice. Instead, they show a mild effect on skeleton development and demonstrated a role for MMP-2 in forming and maintaining the osteocytic canalicular network, and propose that osteocytic network formation is a determinant of bone remodeling and

mineralization. The *Mmp2*$^{-/-}$ mice showed decreased bone mineral density in the limb and trunk bones but increased bone volume in the calvariae. In the long bones, there was moderate disruption of the osteocytic networks and reduced bone density throughout life, whereas osteoblast and osteoclast functions were normal. In contrast, aged but not young *Mmp2*$^{-/-}$ mice had calvarial sclerosis with osteocyte death. Severe disruption of the osteocytic networks preceded osteocyte loss in *Mmp2*$^{-/-}$ calvariae [33]. The difference between the phenotype of *Mmp2*$^{-/-}$ mice and MMP-2 mutations in human was explained by a possible redundancy of Mmp-2 gene function in the mouse. Because *Mmp14*$^{-/-}$ mice phenotype closely resembles that of people deficient in MMP-2 [34, 35], it has been suggested that Mmp-14 in the mouse serves a function similar to MMP-2 in human skeletal development [26]. Another possible explanation for the mild skeletal defects and no focal osteolysis in the *Mmp2*$^{-/-}$ mice is the existence of a modifier gene that modulates the severity of the phenotype. Genetic modifiers are genes that interact with a phenotype-associated gene and alter the observed phenotype. It is possible that there are genetic modifiers of MMP-2, which have co-segregated with the MMP-2 mutations in the affected individuals, and that such genetic modifiers are responsible for the reported different phenotypes of MMP-2 null humans and *Mmp2*$^{-/-}$ mice. Indeed, mice that concomitantly are deficient for *Mmp-2* and carry a mutant that render type I collagen resistant to collagenase-mediated cleavage (*Col1a1*$^{r/r}$ mice) show skeletal changes resembling those reported in the human skeletal syndromes associated with inactivating mutations in MMP-2. Thus type I collagen is an important modifier gene for Mmp-2 during skeletal development in the mouse, and it is possible that minor mutations in type I collagen or in enzymes involved in its processing, which would go largely unnoticed on their own, could result in severe disease in the absence of MMP-2 activity. This study is also consistent with the clinical findings that absence of MMP-2 activity results in progressive bone loss [36].

In 2007, Mosig et al. reported that *Mmp2*$^{-/-}$ mice display attenuated features of human NOA including osteopenia, joint defects consistent with an underlying arthritis, progressive loss of bone mineral density, articular cartilage destruction and abnormal long bone and craniofacial development. These changes were associated with markedly and developmentally restricted decreases in osteoblast and osteoclast numbers in-vivo. Moreover, *Mmp2*$^{-/-}$ bone marrow cells were unable to effectively support osteoblast and osteoclast growth and differentiation. These findings demonstrate that MMP-2 acts not merely as a matrix degrading enzyme, but plays a crucial role in osteoblast and osteoclast behavior and development. These important findings also clarify the paradox of 'How does loss of a proteolytic enzyme result in a 'vanishing bone' disorder?' The loss of MMP-2 probably results in severe osteolysis due to its effects on cell behavior rather than matrix degradation [37].

Deletion of Mmp-2 also demonstrates the role of this enzyme in maintaining bone architecture and quality. *Mmp2*$^{-/-}$ mice show decrease in many of the bone's characteristics, including: connectivity density of trabeculae, mineralization density of trabeculae, strength of vertebra, mineralization density of diaphysis, strength of diaphysis and tissue hardness, together with increase of cortical porosity. This indicates that the loss of MMP-2 weakens the bone [38].

4.2 Mmp-9 Null

Characterization of the *Mmp9*$^{-/-}$ mice demonstrated that MMP-9 is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. These mice revealed no obvious phenotype in the skull or axial skeleton, but their long bones (tibia and femur) were 10 % shorter. This resulted from delay in endochondral ossification of the growth plate. The lack of MMP-9 did not affect chondrocyte proliferation and hypertrophic differentiation; however it caused an expanded area of hypertrophic cartilage, delayed chondrocyte apoptosis coupled with delayed vascularization and ossification. It was suggested that MMP-9 induce apoptosis of hypertrophic chondrocytes which generates an angiogenic signal and leads to vascular invasion. Alternatively, ECM degradation by MMP-9 enables the vascular invasion which in turn causes apoptosis of the chondrocytes in contact with invading vessels [39].

Studies in metatarsals from *Mmp9*$^{-/-}$ and *Mmp9*$^{+/+}$ embryos show that the MMP-9 is essential for recruitment of the very first osteoclasts in these bones. MMP-9 specifically regulates proteolysis of nonmineralized cartilage and release of ECM-bound VEGF, exerting direct chemotactic activity on osteoclasts. In addition, while MMP-9 has almost no collagenolytic activity by itself, it renders the collagenolytic activity of MMP-13 twice as efficient. This collagenolysis-enhancing effect of MMP-9 may favor the penetration of the osteoclasts into the cartilage core of the diaphysis [14].

Later it was discovered that galectin-3, a substrate of MMP-9, is a downstream regulator of MMP-9 activity during endochondral ossification. In *Mmp9*$^{-/-}$ mice galectin-3 is accumulated in hypertrophic chondrocytes due to decreased degradation. This ectopic extracellular galectin-3 delays osteoclast recruitment and removal of hypertrophic chondrocytes at the chrondro-osseous junction. Treatment of wild-type embryonic metatarsals in culture with full-length galectin-3, but not galectin-3 cleaved by MMP-9, mimicked this embryonic phenotype of *Mmp9*$^{-/-}$ mice. This demonstrates that galectin-3 acts as downstream regulator of MMP-9 function at two distinct points during endochondral bone formation, differentiation of late hypertrophic chondrocytes and control of the recruitment of osteoclasts to the front of ossification [40].

In contrast to the weakening effect of the loss of MMP-2, in *Mmp9*$^{-/-}$ mice bone strength was not different from wild type (WT), however bone toughness was decreased. Only few bone characteristics were influenced, in particular: mineralization density of trabeculae and periostal circumference were decreased while connectivity density of trabeculae and brittleness of diaphysis were higher in the *Mmp9*$^{-/-}$ mice compared to WT. Although MMP-2 and MMP-9 have similar substrates, they seem to have different effects on the biomechanical properties of bone. These deferential effects could be due to differences in which bone cell express these two MMPs; MMP-9 is associated with osteoclast migration and angiogenesis while MMP-2 is expressed primarily by osteoblasts and plays a role in canaliculi formation [38].

4.3 Mmp-13 Null

In 2004 two different models of *Mmp13*$^{-/-}$ mice were generated and described for their bone phenotype. Both models demonstrated the role of this enzyme in endochondral ossification and growth plate development. The mouse generated by Inada et al. was a full knock-out, with Mmp-13 deficiency in all tissues. This mouse had a wider growth plates with longer hypertrophic zone. In addition, the chondrocyte columns were less well aligned and were disordered compared with those in WT mice. The *Mmp13* $^{-/-}$ mice showed defects in the development of primary ossification centers together with impaired vascularization. The proposed mechanism for these defects suggests that MMP-13 is normally the collagenase responsible for collagen degradation in growth plate cartilage during endochondral ossification. The excess ECM proteins sequester VEGF accounting for the improper vascularization [41].

The group of Stickens et al. generated mice deficient for MMP-13 in all tissues, as well as mice lacking MMP-13 in a tissue-specific manner in chondrocytes (under control of the *Col2A1* promoter) or osteoblasts (under control of the *Col1α1* promoter). In the *Mmp13* $^{-/-}$ mice chondrocytes proliferate and differentiated normally but their exit from the growth plate was delayed, resulting in lengthened hypertrophic zone with more intense Safranin O staining probably due to decreased aggrecan degradation. Aggrecan and collagen type II were identified as MMP-13 substrates in synergy with MMP-9. In addition, *Mmp13*$^{-/-}$ mice had increased trabecular bone, due to the absence of MMP-13 expression in osteoblasts. Mice with conditional inactivation of MMP-13 in osteoblasts revealed a normal zone of hypertrophic cartilage but an increase in trabecular bone volume proving that increased trabecular bone occur independently of the improper cartilage ECM degradation in late hypertrophic chondrocytes [42].

4.4 Mmp-9; Mmp-13 Null

Mice lacking both MMP-13 and MMP-9 had severely impaired endochondral bone. They display a dramatic alteration in growth plate architecture, namely an expanded hypertrophic zone due to delayed exit of terminally differentiated hypertrophic cells that was more severe than the growth plate phenotype of either the *Mmp9*$^{-/-}$ or the *Mmp13*$^{-/-}$ mice. The normal columnar architecture was lost in the overpopulated *Mmp9*$^{-/-}$; *Mmp13*$^{-/-}$ hypertrophic zone, while the front of ossification was altered similarly but more severe than that observed in the *Mmp9*$^{-/-}$ mice. In addition, these mice displayed diminished ECM remodeling, prolonged chondrocyte survival, delayed vascular recruitment, decreased and defective trabecular bone formation resulting in drastically shortened bones and delayed development of the secondary ossification site. This study indicates a strong synergy between these enzymes in the formation of primary and secondary ossification centers during endochondral bone development, as well as in trabecular bone formation [42].

4.5 MT1-MMP Null

The membrane-bound matrix metalloproteinase MT1-MMP deficiency illustrates its indispensable role in stromal remodeling and in the development, growth, and maintenance of connective tissue structures throughout the body. Both membranous and endochondral ossification are damaged in the absence of MT1-MMP; the ossification of calvarial bones was delayed and suture closure was never completed with evident cranial dysmorphism. The limb bones appeared shorter. Osteopenia was apparent and bone mass was severely reduced as a result of increased bone resorption together with reduce bone formation. Severe generalized arthritis was evident. All joints showed overgrowth of hypercellular, vascularized synovial tissue and destruction of articular cartilage, resulting in ankylosis. Osteoclasts were prominent within articular and periarticular soft tissues. Tendons, ligaments, synovial capsules, musculo-tendinal junctions, and septal/fascial structures associated with skeletal muscle all displayed increased cell proliferation and vascularity, and became increasingly fibrotic. The development of the secondary ossification center was disrupted in MT1-MMP-deficient mice due to failure of blood invasion into the uncalcified epiphyseal hyaline cartilage. This led to growth-plates abnormalities including thinning, disorganization, and lack of chondrocyte proliferation. In addition, MT1-MMP-deficient marrow stromal cells possess two seemingly related defects: impairment of osteogenic capacity and impairment of collagenolytic/gelatinolytic activity [34].

Detailed investigation of the growth plates from MT1-MMP deficient mice demonstrated a three- to fourfold increase in the thickness of the hypertrophic zone which is attributed to delayed resorption of cartilage, while the proliferative zone was disorganized with decreased cell proliferation. In addition, a defect in vascular invasion of cartilage, both during endochondral ossification of growth plates and in secondary ossification centers of chondro-epiphyses was described in these mice, demonstrating that MT1-MMP plays a crucial role in the initial step of angiogenesis [35].

In 2003, Holmbeck et al. presented a novel mechanism of tissue remodeling in the postnatal mouse skeleton. They showed that in the postnatal skeleton, cartilage remodels into bone at multiple sites via a non-endochondral mechanism; i.e., without progression through the sequence of matrix mineralization and osteoclastic removal. The new described process involves coordinated dissolution of the unmineralized cartilaginous matrix by a process that is absolutely dependent on the MT1-MMP and timely apoptosis of nonhypertrophic chondrocytes. This mechanism also allows remodeling of cartilage into ligament at specific sites. Disruption of this mechanism in MT1-MMP–deficient mice results in severe skeletal pathology. This different mechanism of cartilage remodeling was first identified in calvarial cartilage: during embryonic development and until 10 days post natal, the development of membranous bone coincided with the presence of cognate cartilage structures which gradually disappeared as ossification proceeded. The removal of calvarial cartilage anlagen did not involve the conventional sequence of chondrocyte hypertrophy, matrix mineralization, and osteoclastic resorption that characterizes

endochondral bone formation. Instead, the entire anlage remained un-mineralized, populated with type II collagen-expressing chondrocytes and free of osteoclasts. Eventually, these chondrocytes began to express MT1-MMP mRNA in a highly spatially restricted pattern and frequent apoptosis of chondrocytes was detected in the same region. Thus, expression of MT1-MMP in specific regions of the parietal cartilage coincided spatially with cessation of type II collagen expression, with gradual disappearance (regression) of the matrix, and with apoptotic demise of chondrocytes. These changes stand in sharp contrast to the sequence of events that characterize the fate of cartilage during endochondral ossification in long bones, and define a precise and highly regulated program for the removal of un-mineralized cartilage anlagen during the organogenesis of the cranial vault. Additional sites in which these set of MT1-MMP-dependent events occur are: remodeling of the posterior portion of Meckel's cartilage into bone and ligament, matrix remodeling at the ligament–cartilage, muscle–tendon and tendon–bone transitions, as well as in the groove of Ranvier, a zone of direct transition between un-mineralized cartilage and the leading edge of periosteal (perichondral) ossification in long bones. In MT1-MMP–deficient mice this process is blocked due to lack degradation of the anlage. This results in the persistence of distinct, devitalized, ghost cartilages, which retain the shape and anatomical position of the original cartilages, but are noted for a wealth of empty chondrocyte lacunae, and a complete loss of proteoglycan content, highlighting the strict dependence of this mechanism in MT1-MMP and shed light on the phenotype of MT1-MMP deficient mouse [43].

5 MMP's Role in Bone-Related Pathologies

As described so far, MMPs play a critical role in bone development due to their "basic" function as matrix degrading enzymes together with their regulatory function in the availability of bioactive molecules, regulation of cell-matrix interaction, as well as cell proliferation, differentiation, apoptosis etc. Hence, it is not surprising that MMPs take part in many bone-related pathologies part of which are summarized in this chapter and Table 2.

5.1 MMPs in Bone Cancer

ECM degradation is central to malignant tumor growth, invasion, metastasis and angiogenesis. MMPs play a pivotal role in the progression and pathogenesis of many types of tumors, including bone tumors, as well as metastasis into bone. Osteosarcoma (OS) is the most frequent primary bone malignancy among children and adolescence and comprises approximately 35 % of all the bone cancers [44]. MMP-9 [45], MMP-2, and MT1-MMP have been identified in human OS, and were

Table 2 Role of MMPs in bone health and disease

	Development	Cancer	Osteoarthritis	Fracture
MMP-2	Produced mainly by osteoblasts and osteocytes	Expressed in osteosarcoma and associated with poor prognosis Participates in the bone resorption activity of Giant cell tumor of bone Contribute to metastases of breast cancer cells in bone	Increased in osteoarthritic human cartilage	Participates in the inflammatory and cartilage calluses formation stages Important for bone remodeling but not cartilage remodeling during fracture repair Supports the reestablishment of the mechanical properties of the bone matrix
MMP-9	Produced mainly by osteoclast and endothelial cells Essential for osteoclast migration and invasion Regulates proteolysis of non-mineralized cartilage Release ECM-bound VEGF At the chondro-osseous junction: solubilize the type II collagen together with MMP-13 to allow invasion by capillaries	Expressed in osteosarcoma and associated with poor prognosis Essential for Osteosarcoma cell invasiveness Involved in chondrosarcoma cell migration Participates in the bone resorption activity of Giant cell tumor of bone Contribute to prostate tumor growth in the bone microenvironment	Increased in osteoarthritic human cartilage	During the inflammatory stage is expressed by mesenchymal and inflammatory cells surrounding the fracture site In the cartilage callus, expressed at the fracture site Mediates vascular invasion of the hypertrophic cartilage callus Up-regulated during the beginning of the hard callus formation Then expressed by osteoclasts between the hypertrophic cartilage and the newly forming bone and adjacent to sites of vascular invasion
MMP-13	Produced by peri-osteoclastic osteocytes, osteoblasts and hypertrophic chondrocytes Participate in bone resorption	Involved in chondrosarcoma cell migration Important for the bone resorption activity of Giant cell tumor of bone Contributes to tumor-induced bone osteolysis Participates in establishment and progression of bone metastases	Produced by chondrocytes and synovial cells The primary collagenase in osteoarthritis Cleavage and denaturation of type II collagen in articular cartilage	Up-regulated during the beginning of the hard callus formation Participates in degradation of cartilage matrix, resorption and remodeling of the callus
MT1-MMP	Produced mainly by osteoclast, but also in all zones of the growth plate and osteoblasts Allow osteoclasts movement or attachment Activate proMMP-2 and proMMP-13	Expressed in osteosarcoma and associated with poor prognosis		Participates in the inflammatory and cartilage calluses formation stages
Other MMPs	MMP-1, 10, and 11 are expressed in hypertrophic chondrocytes and osteoblasts	MMP-7 is important for tumor-induced osteolysis	MMP-16 and 28 are increased in osteoarthritic human cartilage MMP-1, 3, and 10 are reduced	MMP-8 and 19 are down-regulated over the whole process MMP-7 and MMP-12 inhibit bone healing process

associated with poor prognosis [46]. MMP-8, MMP-13, and MMP-26 were also detected in sarcoma cells [47].

MMPs influence many aspects of cancer progression: MMP-9, in an ERK5-dependent mechanism, is essential for OS cell invasiveness [48]. When induced by Chemokine ligand 2 (CCL2), MMP-9 was found to be involved in chondrosarcoma cell migration via a NF-κB-dependent pathway [49]. MMP-13, induced by either CCN3 or Endothelin-1, is also involved in chondrosarcoma cell migration through the same pathway [50].

Giant cell tumor of bone is an aggressively osteolytic primary bone tumor that is characterized by the presence of abundant multinucleated osteoclast-like giant cells, hematopoietic monocytes, and distinct mesenchymal stromal cells from osteoblastic origin [51]. It has been shown that interleukin-1β and tumor necrosis factor-α, secreted from the giant cells up-regulate the expression of MMP-2, 9, and 13, through their corresponding receptors on the stromal cells. These MMPs, and in particular MMP-13 are important for the bone resorption activity of this tumor [52].

In addition to their activity in ECM break-down, MMPs also regulate other aspects of tumor cells. For example, MMP-2 produced by osteoblasts, significantly contribute to metastases of breast cancer cells in bone by protecting cancer cell against apoptosis and promoting their survival by regulating the bioavailability of TGF-β, a factor critical for cell-cell communication in the bone [53]. Osteoclast-derived MMP-9 affects prostate tumor growth in the bone microenvironment by contributing to angiogenesis through regulation of VEGF bioavailability without altering tumor–induced osteolytic or osteogenic activity [54].

Bone is a major target for metastases in the most frequent solid tumors such as breast, prostate, and kidney cancers. Tumor cells tend to induce an osteolytic phenotype which results from an interaction between the cancer cells and the stromal cells in the bone microenvironment. Microarray analysis investigating gene expression profiling at the tumor-bone (TB) interface identified up-regulation of MMP-13 in both tumor cells and osteoblasts at the TB interface. MMP-13 expression contributes to the osteolytic process by regulating RANKL/OPG levels, activating MMP-9, and increasing TGF-β signaling [55]. In addition, MMP-13 contributes to pre-osteoclast differentiation by promoting the cleavage of galectin-3, a suppressor of osteoclastogenesis [56]. The ability of breast cancer cells to induce MMP-13 expression in osteoblast, and the key role of MMP-13 in the establishment and progression of bone metastases was demonstrated in further studies [57]. Recently it was shown that treatment with an MMP13-selective inhibitor Cmpd-1 reduced the growth rate of two different primary breast cancer models, delayed their onset of development and reduced the severity of tumor-induced osteolytic lesions in experimental xenograft model of bone metastasis following intra-cardiac inoculation of breast cancer cells [58]. MMP-7 was also identified as an important contributor for tumor-induced osteolysis. It has been shown that osteoclast-derived MMP-7 promotes RANKL solubilization in the tumor-bone microenvironment thus promoting osteoclast precursor recruitment and maturation [59].

5.2 MMPs in Osteoarthritis

Osteoarthritis (OA) is a chronic degenerative joint disease causing disability in the elderly, pain, stiffness and loss of function in articulating joints. OA is characterized by changes in the anatomy of load-bearing joints that lead to degradation of articular cartilage, inflammation of the synovium (synovitis), changes to subchondral bone and growth of new bone and cartilage (osteophytes) at the joint edge. The causes of OA are not fully understood, but mechanical factors such as joint injury and obesity are thought to be primary initiators of disease, with other risk factors such as age, gender and genetics, which all contribute to disease development and progression [60].

Cartilage degradation is fundamental in the pathogenesis of OA. During this pathogenesis, MMPs are secreted by chondrocytes and synovial cells including fibroblasts and macrophages. MMP-13 is considered as the primary collagenase in OA. Its production by chondrocytes is increased in OA [61] and it is involved in the augmented cleavage and denaturation of type II collagen in articular cartilage during OA [62]. Over-expression and excessive MMP-13 activity in hyaline cartilages and joints of mice results in articular cartilage degradation and joint pathology of the kind observed in OA [63], while MMP-13 deficient mice are resistant to osteoarthritic cartilage erosion [64].

Expression profile of MMPs revealed increase in the expression of MMP-2, 9, 13, 16, and 28 in osteoarthritic human cartilage isolated from femoral heads compared to normal cartilage, while the expression of MMP-1, 3, and 10 was reduced. The reduced expression of MMP-1 may suggest that this collagenase has no role in cartilage collagen degradation in OA of the hip, or that its role is earlier in the disease process [65].

Several factors have been shown to induce MMP expression during OA (reviewed by Troeberg and Nagase): the pro-inflammatory cytokines IL-1β, Hypoxia-inducible factor 2α (HIF-2α), Histone deacetylases, mechanical stimulation, extracellular sulfatases, discoidin domain receptor-2 (DDR-2), protease-activated receptor 2 (PAR-2), Wnt signaling, and miR-22 [60]. High Body Mass Index (BMI) is also considered a risk factor for OA. Epidemiological studies have shown that the risk for knee osteoarthritis is increased by 36 % for every 2 units of BMI. It was shown that both high BMI and inflammation induce MMP-13 expression in osteoarthritic cartilage through elevation of microRNA-22 (miR-22), which in turn inhibits the expression of Bone morphogenetic protein-7 and Peroxisome proliferator-activated receptor alpha. Reduction in these two factors increases the expression of IL-1β which induces the expression of MMP-13 in osteoarthritic cartilage [66]. These findings demonstrate the complex gene networks regulating MMP expression in OA.

5.3 MMPs and Fracture Repair

Fracture healing is a complex event which is typically characterized by four overlapping stages: the initial inflammatory response, soft callus formation, hard callus

formation, initial bony union and bone remodeling [67]. During the repair process a large amount of cartilage is formed and rapidly replaced by osseous tissue. Hence, the ECM undergoes dynamic changes as collagen type II is being replaced by collagen type I. Large scale analysis identified up-regulation of several MMPs during fracture repair. Interestingly, the MMPs could be divided into two groups according to their temporal regulation during the process: MMP-2 and MT1-MMP were increased after the formation of the fracture and until day 10 post-fracture. This represents the inflammatory stage (day 3) up to the peak of cartilage calluses formation (day 10). On the other hand, MMP-9, and MMP-13 were up-regulated approximately on day 10. MMP-8 and MMP-19 were down-regulated over the 21 days of bone healing. MMP-8 is expressed predominantly by neutrophils, hence its down-regulation suggests that this cell type is largely absent in the healing tissues, until the marrow element has been reestablished during the late phases of primary bone formation [68]. MMP-19 is also related to the immune system. It is expressed in macrophages [69, 70] and is up-regulated under inflammatory conditions such as arthritis and multiple sclerosis [71–73].

Other study had located MMP-13 in the healing fracture from day 1. It demonstrates the crucial role of MMP-13 in the repair process starting from the periosteum at day 1 post fracture, throughout its expression in the cartilaginous callus, and later in the reparative and remodeling phases in which it is expressed by both hypertrophic chondrocytes and immature osteoblasts. At this stage, MMP-13 initiates the degradation of cartilage matrix, resulting in resorption and remodeling of the callus [74]. In line with that, MMP-13 deficient mice had significantly delayed fracture repair, characterized by a retarded cartilage resorption in the fracture callus and defects in vascular penetration and chondroclast recruitment to the callus suggesting that MMP-13 is vital to the process of angiogenesis during healing of fracture, especially in the cartilage resorption process [75].

MMP-9 is abundantly expressed during the inflammatory stage in mesenchymal and inflammatory cells surrounding the fracture site. In the cartilage callus, it is expressed at the fracture site and during the latter stages it is detected in osteoclasts/chondroclasts between the hypertrophic cartilage and the newly forming bone of the fracture callus and adjacent to sites of vascular invasion. MMP-9 mediates vascular invasion of the hypertrophic cartilage callus, probably by regulating the bioavailability of *VEGF*. *Mmp9*$^{-/-}$ mice have non-unions and delayed unions of fractures caused by persistent cartilage at the injury site.

In contrast, MMP-2 is important for bone remodeling but not cartilage remodeling during fracture repair. It is diffusely expressed at the fracture site and in the surrounding soft tissues immediately adjacent to the fracture site as early as day 3 post-fracture and later its expression becomes stronger in areas of the callus in which cartilage and bone form. Bone remodeling in the late stages of repair is essential to restore the structural and mechanical properties of the broken bone. MMP-2 might support the re-establishment of the mechanical properties of the bone matrix at these stages [76]. The expression of MMP-2, 9, 13, and MT1-MMP during fracture repair is regulated, at least in part, by TNF-α which coordinate the expression of regulators of endothelial cell survival and metalloproteolytic enzymes and is essential in the transition and progression of the endochondral phase of fracture repair [77].

Failure of healing a broken bone is called nonunion. MMP-7 and MMP-12 are highly regulated at nonunion tissue compared with local mineralized callus from the same site. Both MMP-7 and MMP-12 can directly bind to and degrade BMP-2, the best established growth factor in bone formation and repair. It is suggested that these two enzymes inhibit the healing process through the inactivation of BMP-2 [78].

6 MMPs in the Vascularization of the Avian Growth Plate

Two skeletal abnormalities in avian species, Tibial dyschondroplasia (TD) and rickets result from impaired bone ossification and vascularization [79–81] and can serve as a good models for studying process and genes involved in matrix mineralization, assembly, and calcification [82–85]. The involvement of MMPs in the etiology of these syndromes shed light on their function in the growth plate. MMP-2 is linked to chondrocyte differentiation in the avian growth plate; it is over-expressed in the rachitic lesion and is probably involved in maintaining the chondrocytes in proliferative state in this syndrome [86]. In both lesions, the expression of MMP-3, 9, and 13 is reduced (in TD lesion MMP-2 is also reduced) and is associated with the expulsion of blood vessels from the lesion's area indicating their involvement in vascularization of the growth plate [86, 87]. In accordance, during recovery from TD, MMP-2, 3, 9, and 13 reappear in the growth plate, in parallel to the renewed vascularization [87].

We used the avian growth plate as a model for studies of mechanical load since like humans, chicken stand on two legs as opposed to most mammalian species used as experimental models [88]. Mild mechanical load (addition of 10 % of body weight in young chicks) as well as release from load and the consequent increased vascularization and catch-up growth, caused increase in the expression of MMP-2, 9, and 13 in the growth plate. Interestingly, MMP-3 was reciprocally regulated by load and load-release: both MMP-3 and its associated protein connective tissue growth factor (CTGF) were down-regulated by load but up-regulated following release from load. In opposed to MMP-2, 9, and 13, MMP-3, is probably involved in the maintenance of the cartilage itself, and not in the vascularization process and exert its actions through CTGF [89].

7 Conclusions

No doubt MMPs play a significant role in bone development in both health and disease and are necessary for normal bone growth and strength. The main MMPs involved in bone resorption and endochondral ossification are illustrated in Fig. 1. One interesting aspect arising from studying the involvement of this family of enzymes in bone is the fact that out of 28 family members only 4 (MMP-2, 9, 13, and MT1-MMP) are expressed and dominant in different stages of bone development

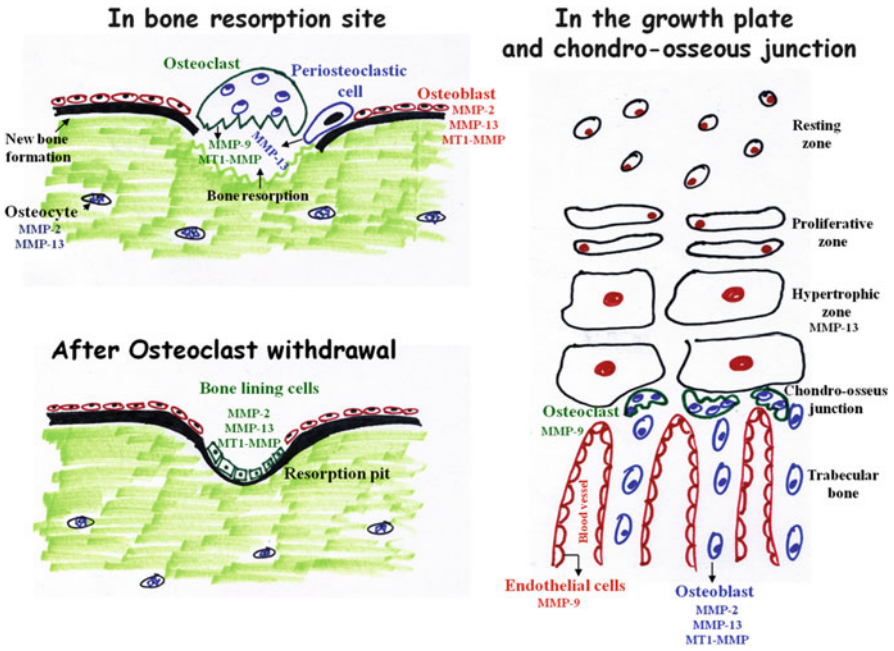

Fig. 1 The main MMPs involved in bone resorption and endochondral ossification. During bone resorption MMP-9 and MT1-MMP are secreted by osteoclasts. However, these enzymes do not participate in matrix resorption per se but rather in osteoclasts migration and invasion. In contrast, MMP-13 is secreted from peri-osteoclastic osteocytes, translocate from the osteocytes into the resorption site where it participate in resorption. After osteoclast withdrawal, bone lining cells enter the lacuna and secrete MMPs (probably MMP-2, 13, and MT1-MMP) which remove demineralized collagen that was not digested by the osteoclasts. This "cleaning" is prerequisite for the subsequent deposition of a first layer of collagenous proteins in the resorption pit. MMP-9 and MMP-13 are the most important MMPs in endochondral ossification. MMP-13 is secreted from hypertrophic chondrocytes and MMP-9 from osteoclasts and endothelial cells

including blood invasion, osteoclast function, ECM degradation, formation of canaliculi etc. The importance of normal function of each one of these enzymes is especially demonstrated in the bone-related syndromes characterizing the human mutations in MMP-2, 13 and MT1-MMP. This further emphasizes that the lack of one MMP in bone can't be compensated by other members of the MMP family.

Is it possible that MMPs regulate process in bone by inducing yet-unknown signaling? Two facts strength this suggestion: First, the fact that MMPs are not significant for the resorption of long bones [11], yet mutations in MMPs or MMP deficiency strongly affect their development, suggest that MMPs play a role which is other that matrix degradation. Second, the existence of MMP-induced signaling which does not work through ECM degradation can explain the paradox of "the vanishing bone syndromes"—how the absence of a single protease causes osteolysis rather than osteopetrosis. This arises an important question- does knock out

models are the best strategy to understand MMP activity? Maybe targeted mutations in different domains of the MMP protein can shed light on other functions of these enzymes besides proteolysis?

For example, it is well known that MMPs regulate signaling events by processing ligand and hormones, cytokines, chemokines, adhesion molecules and other membrane receptors. However, MMPs also cleave intracellular substrates within cells in nuclear, mitochondrial, various vesicular and cytoplasmic compartments, including the cytoskeletal intracellular matrix. Their substrates include apoptotic regulators, signal transducers, molecular chaperones, cytoskeletal proteins, systemic autoantigens, enzymes in carbohydrate metabolism and protein biosynthesis, transcriptional and translational regulators, and proteins in charge of protein clearance such as lysosomal and ubiquitination enzymes [90]. These localizations, although not completely understood, open a new field in MMP studies, aiming to find new functions and down-stream targets.

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Escalated Expression of Matrix Metalloproteinases in Osteoarthritis

Srijita Dhar, Bimal K. Ray, and Alpana Ray

Abstract Osteoarthritis (OA) is a disease that damages the joint tissues and often destroys the cartilage lining of the joint tissue irreversibly. This leads to loss of joint function making movement difficult and crippling the affected individuals. OA primarily affects elderly population and with increasing aging human population in the world it is going to affect a very large portion of the population in the near future. Unfortunately there is no current method of treatment of this disease except by suppressing the pain with pain-killer and occasional use of steroid to reduce excruciating pain. More radical treatment and often the only remaining option is surgery where affected joint tissue is removed and replaced with artificial prosthetics embedded into the bone, a procedure called arthroplasty. The surgical procedure is costly and sometimes associated with recurring post-operative problems. New approaches to control and cure OA could come from a better understanding of the cellular and subcellular events in the joint tissues of OA patients. Since cartilage depletion is a major pathological event in the progression of OA, much attention has been given to the cellular mechanisms responsible for the cartilage damage. This review will address this cellular process by focusing on enzymes which are activated in the cartilage tissue and cause its depletion.

Keywords Osteoarthritis • Cartilage • Chondrocytes • Gene expression • Transcription factors • Molecular interactions • Transcriptional regulation

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1 Introduction

Etiology of OA has been extensively studied, which has revealed that repetitive use of the joints is a major factor in the development of OA. Although OA was earlier regarded as a disease of the wear-and-tear, it is now realized that inflammation and synovitis is present in a significant percentage of patients. Studies on the cartilage degeneration led to the discovery of specific proteases, matrix metalloproteinases (MMPs) and their natural inhibitors (TIMPs) which have been linked to the root cause of cartilage degeneration. These important discoveries have paved the way for new therapeutic intervention to control cartilage damage.

2 Matrix Metalloproteinases in OA

As a family of proteases, matrix metalloproteinases (MMPs) play an important role in the maintenance of cartilage by selectively degrading extracellular matrix for regeneration of the cartilage [1, 2]. The resident chondrocyte cells of the cartilage are one of the major sites for the synthesis of these highly specific proteins [3, 4]. During the pathogenesis of OA, these proteases become highly destructive causing massive damage to the cartilage. Since the functional role of MMPs in cartilage degradation will be discussed in several accompanying articles in this review series, we will primarily focus on the expression of these molecules in the affected tissues and cells in OA.

3 Escalated Expression of MMPs

Many studies have reported that high level of MMPs in arthritic cartilage is a consequence of the over-expression of the genes coding for these degradative enzymes by transcriptional induction and have been reviewed [5, 6]. Chondrocyte cells of the cartilage and the synovial fibroblasts are two cell types that undergo changes in the regulatory mechanism for the expression of MMP genes and thus allowing massive accumulation of these highly active proteases [7, 8].

Understanding of the molecular mechanisms responsible for increased expression of MMPs in the cartilage-specific cells of OA joint tissue has been the focus of many studies and is critical to find an effective treatment and a possible cure for joint disease. Many studies in recent years have focused on different transcription factors which are the major driving force in the escalation of expression of MMP genes. Several transcription factors have been linked to the induction of MMPs in different cell types and these regulatory proteins include AP1, AP-2, Sp1, C/EBP, NF-κB, NF-1, PEA3, Runx-2, SAF-1 [7, 9–14].

4 Role of SAF-1 in MMP Expression

As an inflammation-responsive transcription factor, SAF-1 has been found to regulate several MMP genes including MMP-1, MMP-9 and MMP-14. SAF-1 directly binds to the specific promoter elements in these MMP genes and such interaction requires activation of SAF-1 by different signal transducing kinases such as protein kinase A, protein kinase C, MAK kinase [15–17]. High level of SAF-1 has been identified in human OA patients' joint and in the osteophytes [18]. These findings have suggested that SAF-1, in addition to its role in cartilage degradation, might also be involved in osteophyte formation. In this review, we will describe some of the key molecules that are linked to the pathway of osteophyte formation and describe how these molecules function in the affected cartilage and bone in OA pathogenesis.

5 Osteophyte Formation in OA

One important pathophysiological phenomenon of OA is the repair or restoration process by which cells and tissues of the joint attempt the healing process by activating various repair pathways. Most notable event is the reinitiation of bone and cartilage growth to compensate for the loss of these tissues during the degenerative event of the disease process. An unintended consequence of this new growth event is the development of osteophytes which exacerbates the severity of OA with a very poor prognosis. While this is a serious clinical symptom, relatively little is known about the molecular events associated with osteophyte development. For an effective management of OA pathogenesis it is essential to understand how osteophyte develops. In recent years, scientists are focusing on the biochemical and molecular events associated with this apparently faulty repair process that gives rise to osteophyte formation.

6 Mechanism of Osteophyte Formation: Inappropriate Cartilage Remodeling Due to Re- Initiation of Developmentally Regulated Pathways as a Probable Cause

Central to the development of therapies for the prevention of osteophyte formation is a basic understanding of the development steps along with the identification of the involved factors. Although a general perception is that osteophytes might help stabilizing the joints affected by OA [19], these are the principal cause of joint pain experienced by OA patients. Osteophytes are non-neoplastic cartilaginous

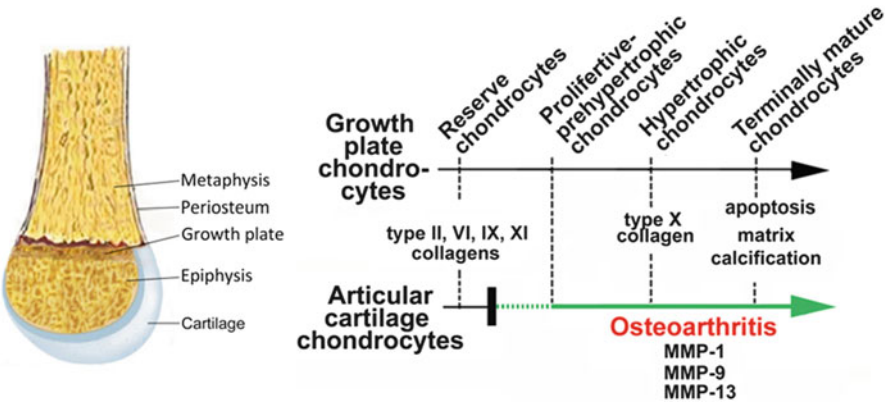

Fig. 1 Stages of chondrocyte differentiation. Growth plate chondrocytes proceed through a tightly regulated pathway leading to terminal maturation (*black horizontal arrow*). Articular cartilage chondrocytes, which derive from same chondrogenic precursor, do not display same gene expression profile as seen in growth plate chondrocytes. A barrier shown by a *thick vertical line* appears to halt any further differentiation of chondrocytes and the normal physiological state of articular cartilage is maintained. When this barrier is breached at the onset of OA-pathology (appearance of the *green line*), articular cartilage chondrocytes show abnormal gene expression profile which somewhat mimics recapitulation of the events seen during endochondral ossification

protrusions growing at the margins of joints and are believed to arise from endogenous repair attempts in the degenerating cartilage. Recent investigations using molecular approaches indicate that a low-level cartilage regeneration process is highly active in the chondrocytes of OA cartilage. Such proliferative activity leads to cloning and clustering of the chondrocytes, a typical feature of OA articular cartilage. Chondrocytes of OA cartilage have been shown to synthesize several structural components of ECM, such as small proteoglycans [20], type IIA and type IIB collagens [4, 21], and type X collagen [22, 23]. Expression of type IIA collagen is indicative of a chondroprogenitor phenotype, which is comparable to the chondroprogenitor phenotype observed in fetal skeletal development [24, 25]. Type X collagen is a signature marker of hypertrophy of growth-plate chondrocytes [22, 26]. These factors are the key to understanding of how to control inappropriate cartilage remodeling and osteophyte formation as seen in osteoarthritis.

While analogy has been drawn between osteophyte formation and growth plate development as a part of endochondral ossification, there is a fundamental difference between the chondrocytes of articular cartilage and the chondrocytes of growth plate cartilage that undergo mineralization to form bone (illustrated in Fig. 1). Although articular and growth plate chondrocytes originate from a common chondroprogenitor cell, growth plate chondrocytes progress though a tightly regulated series of differentiation steps leading to terminal maturation and bone formation that is widely known as endochondral ossification. In contrast, articular chondrocytes express collagens type II, VI, IX and XI that are necessary for the maintenance of the extracellular matrix (ECM). Normal articular cartilage chondrocytes never

express type X collagen and also do not undergo apoptosis and matrix calcification. Phenotypic alteration of OA articular chondrocytes is a highly intriguing phenomenon and it is postulated that reinitiation of some of the cartilage matrix synthesizing activities may cause the formation of osteophytes or bony outgrowths. It appears that a barrier maintaining the chondrocytes in articular cartilage in the proliferative stage is breached. There is yet no report as to what triggers this reinitiation of chondrocyte differentiation in OA.

7 Role of Angiogenesis, Inflammation and VEGF in OA and Osteophyte Development

Angiogenesis and inflammation play a big role in the pathogenesis of OA. These two events are closely integrated as inflammation can stimulate angiogenesis and angiogenesis can facilitate inflammation. Angiogenesis occurs at the osteochondral junction as well as within the OA synovium. In OA, complications resulting from angiogenesis are several. First, increased permeability of newly formed blood vessels to macromolecules facilitates edema or swelling. Second, growth factors that regulate vascular invasion and angiogenesis can promote osteophyte formation, thickening of underlying subchondral bone and development of fibrocartilage. Third, angiogenesis can facilitate pain through structural reorganization of the joint.

Osteophytes which are non-neoplastic cartilaginous and osseous protrusions growing at the margins of osteoarthritic joints involve complex patterns of cellular proliferation, differentiation, as well as matrix synthesis and turnover. Histologic and biochemical examination showed that considerable similarity exists between the proliferation and differentiation pattern of the chondrocytes in the osteophytes and in epiphyseal growth plate [27]. Osteophyte development is closely followed in various experimentally induced animal models [27, 28]. These studies have indicated that neoangiogenesis is an important factor in the initiation of new matrix growth and mineralization. Vascular endothelial growth factor (VEGF) is a major proangiogenic factor involved in promoting angiogenesis in many tissues including the cartilage [29]. In developing growth plates hypertrophic chondrocytes express abundant VEGF which provides signal for capillary invasion, chondrocyte differentiation, apoptosis and ossification of the matrix [30]. In the osteophytes, VEGF expression is detected in the hypertrophic chondrocytes near the newly formed blood vessels at the cartilage bone junction and it is suggested that VEGF secreted from these cells provides a signal for vascular invasion of the cartilage, followed by chondrocyte apoptosis and matrix calcification [28].

Five isoforms of VEGF have been identified that arise due to alternative splicing of VEGF mRNA from the single VEGF gene. VEGF is highly expressed during embryogenesis and development. In adults, VEGF is expressed only in certain instances such as menstrual cycle, would healing, ECM remodeling and during pathological situations. VEGF and its two receptors are almost undetectable in normal adult cartilage. In correlation with its role in osteophyte development, VEGF is seen

to be abundantly expressed in the chondrocytes present at superficial layers at early stages OA and in chondrocytes at the deep zone in severely degraded OA cartilages [31]. Synovial fluids of OA patients contain high levels of VEGF and macrophages harvested from synovial tissue of OA patients are shown to express abundant VEGF [32]. VEGF also induces expression of several MMPs and simultaneously reduces expression of TIMPs [33]. High level of VEGF expression in arthritic joint has been linked to high level of active SAF-1 transcription factor in the cartilage [34].

The possible ways VEGF contribute to the pathogenesis of OA and osteophyte development are several. First, VEGF is implicated in osteophyte formation [28], in the advancement of underlying subchondral bone, and for the development of fibrocartilage [35]. As in growth plates VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation [30], expression of VEGF in OA chondrocytes may be regarded as a de-differentiation process.

8 Role of TGF-β in Osteophyte Formation

Osteophyte development is believed to be a continuous process from pluripotent mesenchymal cells to mature hypertrophic chondrocytes. Mechanical overload is suggested to be a signal [36] but as most osteophytes are found in non-weight bearing areas, considerable skepticism prevented this hypothesis to gain universal acceptance. In contrast the likelihood of the cytokines or growth factors playing a dominant role in osteophyte development gained popularity. TGF-β and members of this family are shown to initiate chondrogenesis and to be markedly expressed by human osteophytes [37–39]. TGF-β is a member of a large family of structurally related cytokines consisting of more than 30 ligand proteins regulating a wide variety of biological processes, including growth, differentiation and development [40]. Consequently TGF-β was described as a cartilage inducing factor. Three distinct isoforms of TGF-β (TGF-β-1, -2, and -3) which are encoded by distinct genes are also functionally distinct. Signaling via TGF-β superfamily is initiated through type I and type II transmembrane serine/threonine kinase receptors. Ligand binding induces the formation or stabilization of a complex and further propagated by involving Smad proteins [41].

In the osteophytes obtained from human patients, TGF-β mRNA expression is seen in the chondrocytes of superficial layer. The level of TGF-β is also seen to be high in synovial joints of OA patients [42]. In correlation with regenerative action of TGF-β, it is present at a very low level in the chondrocytes of degenerative articular cartilage. In animal models, repeated intraarticular injection of TGF-β is shown to induce osteophyte development or enhance osteophyte formation in animals with experimentally induced OA [38, 39, 43]. Adenoviral over-expression of TGF-β also increases osteophyte development [39]. In correlation, inhibition of endogenous TGF-β is shown to reduce the rate of osteophyte development in experimental animal models of OA [44]. BMP-2 a member of the TGF-β superfamily is also shown to induce osteophyte development [43]. Synovial lining macrophages play an important role in osteophyte development and depletion of synovial macrophages marked inhibit osteophyte formation [45]. Apoptosis is another important factor in

controlling osteophyte formation and several studies reported the involvement of nitric oxide in chondrocyte cell death and chondro-osteophyte formation [28, 46]. DNA damage is abundantly seen in the hypertrophic zone.

9 Role of Parathyroid Hormone, Parathyroid Hormone-Related Protein and Its Receptor in the Stimulation of Bone Growth

Parathyroid hormone related protein (PTHrP) was originally identified as a pathogenic factor for malignancy associated hypercalcemia [47, 48]. Further studies indicated that the amino terminal sequences of the parathyroid hormone related protein (PTHrP) and parathyroid hormone (PTH) are similar and both molecules can exert their actions by interacting with a common receptor known as PTHR1 receptor. PTH and PTHrP are evolutionary related peptides in which PTH is produced exclusively in the parathyroid glands and PTHrP is produced in many tissues.

PTHrP is not a hormone but is proposed to act as a paracrine regulator in several tissues including the bone. It has been known for a long time that once daily subcutaneous injection of low level of parathyroid hormone (PTH) extract or PTHrP increases trabecular bone volume in animals and humans [49]. However, when continuous infusion of PTH was given by Alzet osmotic pumps the anabolic effect of PTH/PTHrP was not seen, instead bone resorption occurred [50]. These results suggested that continuous infusion of PTH/PTHrP may have turned on or off many more genes than single daily boluses of the peptide and the action of PTH/PTHrP on bone growth is highly complex. Genetic abnormalities in PTHrP and PTHR1 receptor proteins are shown to cause several human diseases, including Blomstrand's chondrodysplasia [51, 52] and Jansen's metaphyseal chondrodysplasia [53, 54].

Transgenic and knockout mice of PTHrP or PTHR1 genes show severe chondrodystrophy [55–58]. The $PTHrP^{-/-}$ mice die at the time of birth [57] but heterozygous $PTHrP^{+/-}$ mice survive and develop to be osteoporotic [57, 59]. The $PTH^{-/-}$ mice exhibit increased bone volume due to reduced PTH-induced osteoclast formation and bone turnover. Osteoblast-derived PTHrP has been shown to act as a potent endogenous bone anabolic factor that potentiates bone formation by altering osteoblast recruitment [60]. PTHrP is shown to enhance production of receptor activator of NF-κB ligand (RANKL) and thereby promoting osteoclast formation [61] which may explain, at least in part, why sustained elevation of PTH/PTHrP favors osteoclast formation.

10 Role of PTHrP and PTHR1 Receptor in Cartilage and Skeletal Development

PTHrP and PTHR1 play a key role in skeletal development by controlling epiphyseal growth and cartilage mineralization [56]. In the developing growth plate, PTHrP is expressed in the perichondrium and resting and proliferative chondrocytes

while PTHR1 receptor is expressed in proliferative and prehypertrophic chondrocytes [62]. However, the precise mechanisms by which PTHrP/PTHR1 affect skeletal development are less clear. One of the regulators of PTHrP/PTHR1 action is Indian Hedgehog (Ihh) protein and its receptors [62]. It is postulated that when proliferating chondrocytes decide to undergo hypertrophy they express higher levels of PTHR1 receptor. Chondrocytes committed to this pathway then transiently express Ihh, until they become fully hypertrophic. The Ihh protein through an unclarified pathway increases the local secretion of PTHrP. PTHrP then signals back to chondrocytes expressing PTHR1 receptor, thereby preventing nondifferentiated chondrocytes from moving down the hypertrophic pathway.

11 Involvement of SAF-1 in Osteophyte Formation

The abundance of SAF-1 in the osteophytes within the arthritic joints of CMV-SAF-1 transgenic mice [63], in human patients with severe OA [18] and canine ACLT model (Fig. 2) suggested for a role of SAF-1 in osteophyte development.

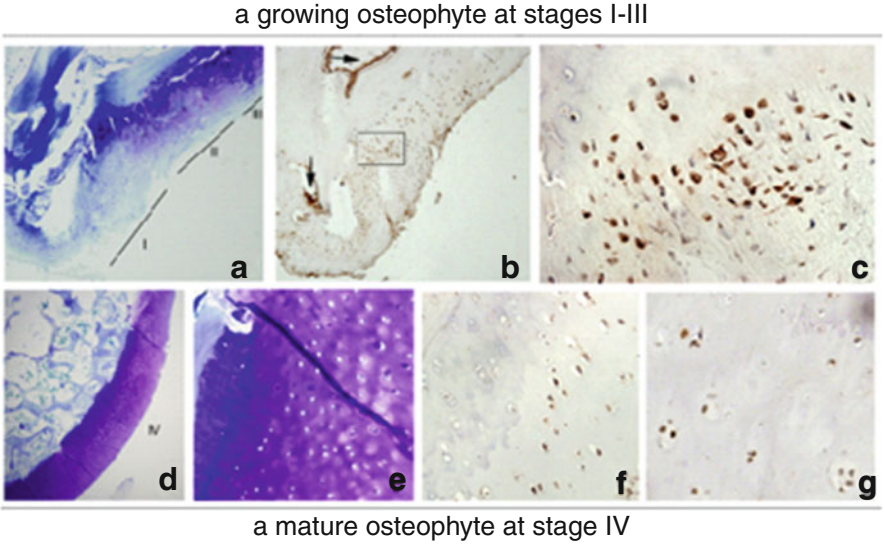

Fig. 2 Expression of SAF-1 in a canine model of osteophyte development (experimentally induced by ACLT). (**a**) Toluidine *blue* staining of a growing osteophyte, between stages I and III. Cells at stage I show little *blue* stain due to less GAG synthesis. As the osteophyte develops GAG synthesis is increased. (**b**) A serial section of the osteophyte is immunostained with anti-SAF-1 antibody. High level SAF-1 expression is detected in the proliferating fibroblastic chondrocytes. (**c**) High magnification view of the boxed region in panel **b**. Also noticeable is strong SAF-1 stain in the new blood vessels that correlates with one of its possible functions as a regulator of VEGF expression (*arrow*, panel **b**). Panels, **d** and **e** show toluidine *blue* stain of a mature osteophyte. (**f**) SAF-1 positive chondrocytes are well visible in the deep layer. Chondrocytes below the tide mark show very little to none SAF-1 expression. (**g**) Higher magnification view of hypertrophic chondrocytes expressing SAF-1

Several key findings substantiate the involvement of SAF-1 in this process. First, osteophyte development is more pronounced in arthritic CMV-SAF-1 transgenic mice as compared to the arthritic non-transgenic littermates. Secondly, arthritic CMV-SAF-1 mice display higher level of angiogenesis, synovial inflammation, infiltration of macrophage cells and cartilage degradation than arthritic non-transgenic littermates [63]. Third, SAF-1 is abundantly expressed in naturally occurring human osteophytes and in the osteophytes in canine model after induction of OA by ACLT. Fourth, SAF-1 promotes cartilage degradation and plays a role in joint structure remodeling by increasing the expression of MMP-1 and -9 [7, 8]. Fifth, SAF-1 function is induced by TGF-β that promotes osteophyte formation. These findings suggest that SAF-1 is a common regulator of the genes that are linked to both cartilage degradation and repairing activities and an imbalance of these two functions, which are controlled by SAF-1, could lead to osteophyte development.

12 Role of SAF-1 in the Pathogenesis of OA

In OA, two opposite acting metabolic events are seen to occur. In the early phases of OA, in response to the degeneration of the cartilage matrix, a biosynthetic phase is stimulated. Articular chondrocytes which are metabolically quiescent become stimulated and undergo cell proliferation and synthesize many of the ECM components. Cloning of chondrocytes to form so called "clustered" chondrocytes and formation of chondro-osteophyte nodules are believed to occur from such response. However, at some point the degenerative activity in OA cartilage becomes higher than the matrix repairing activity and as the disease progresses cartilage degradation becomes the predominant effect. The SAF-1 transcription factor in previous studies was identified as a transcriptional regulator of MMP-1 and MMP-9 proteases that play crucial role in cartilage degradation [7, 8]. However, the detection of SAF-1 in clustered chondrocytes and in the chondro-osteophytes suggests that SAF-1 may have a role in cartilage matrix synthesis. The cartilage matrix repairing event which is seen in OA appears to mimic the endochondral ossification during normal skeletal growth and development. Endochondral ossification is a highly complex process requiring orchestration of chondrogenic and osteogenic events and participation of many gene products. Parathyroid hormone related protein (PTHrP), its receptor and the *Ihh* gene product regulate chondrocyte growth and maturation. SAF-1 most likely regulate cartilage matrix repairing activity as a transcriptional regulator of PTHrP receptor, and this activity of SAF-1 contributes to its role in the delay of chondrocyte differentiation and hypertrophy and prolongs the repair process. These distinct roles of SAF-1 in the degenerative and bone growth pathways have been depicted in a model shown in Fig. 3.

Exact role of SAF-1 in OA-pathology is only beginning to become clear. In OA cartilage an increasing number of hypertrophic chondrocytes, especially chondrocytes adjacent to degraded matrix or lesions, exhibited high level of SAF-1 immunostaining, which suggested that chondrocytes may synthesize SAF-1 before

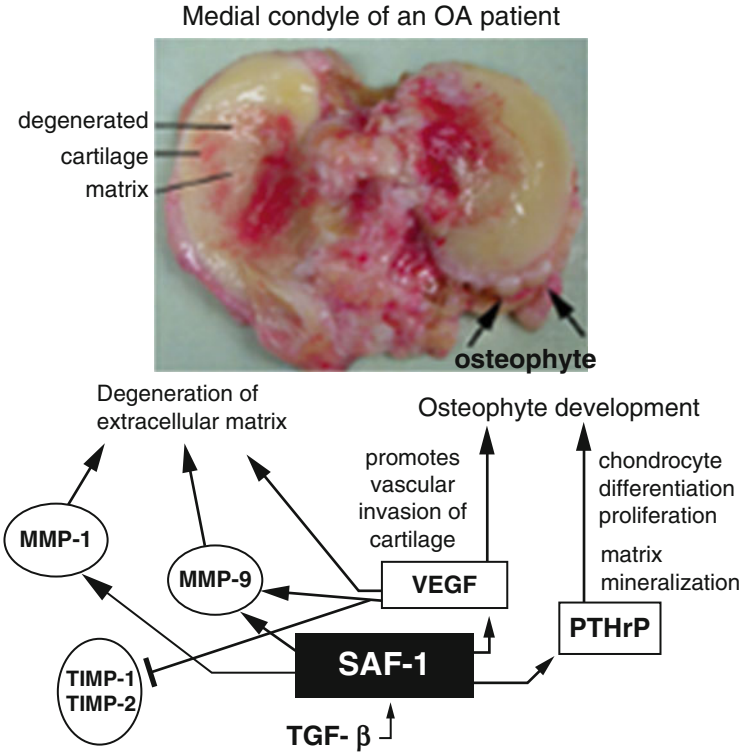

Fig. 3 A model depicting the pathways regulated by SAF-1 in the cartilage and subchondral region of joint tissue in the pathogenic progression of OA

proceeding to apoptosis. Often apoptotic chondrocytes with nuclear condensation at the deep zone are seen to contain SAF-1 protein. This could well be true as SAF-1 is shown to induce apoptosis of cells via induction of p21 cyclin-dependent protein kinase (CDK) inhibitor gene expression in chondrocyte and synoviocyte cells [64]. SAF-1 may have a dual role in the metabolism of OA cartilage, such as promoting cartilage degradation and matrix repair.

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Role of Proteases in Autism Spectrum Disorders

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Abstract Autism spectrum disorders (ASDs) are severe heterogeneous neurodevelopmental disorders. Interaction of genes and environmental factors origin these enigmatic conditions. ASDs are characterized by dysfunctions in social interaction and communication skills, repetitive and stereotypic verbal and non-verbal behaviours. Autistic children show immune dysfunction. ASDs are increasing in incidence and prevalence. Between 1 in 80 and 1 in 240 with an average of 1 in 88 children in the United States have an ASD, according to Center for Disease Control. The mechanisms of ASD pathogenesis are still unknown, it is priority to provide either preventative or corrective therapies. Available treatments for autism can divided into behavioural, nutritional and medical approaches, although no defined standard approach exists. ASDs are increasingly being recognized as a public health problem. The lifetime cost to care for an individual with an ASD is $3.2 million. The lack of a specific biomarker for ASDs rends these syndromes too often underestimated. The priority for the future of ASD management is the identification of

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potential targets for the development of diagnostic and/or therapeutic strategies. Several proteases are involved in the regulatory mechanisms of ASD pathogenesis and maintenance, as well as in cellular functions underlying these disorders (i.e. inflammatory and both innate and adaptive immune responses). Proteases could be an interesting target to better unravel the immune and inflammatory molecular pathways involved in ASD pathophysiology.

Keywords Autism spectrum disorders • Biomarkers • Proteases • Caspases • Matrix metalloproteases

1 Introduction

1.1 Autism and Autism Spectrum Disorders

Autism and autism spectrum disorders (ASDs) are severe, complex and heterogeneous neuro-developmental syndromes [1]. Their exact aetiology, as well as the pathophysiology and defined molecular mechanisms of pathogenesis, remains still poorly understood. Likely, their origins are in the interaction of several genes and environmental factors. Indeed, these pathologies could be referred as multi-factorial and polygenic disorders, as they results from a complex combination of genetic, epigenetic, environmental (i.e. air pollution, organophosphates, heavy metals), and immunological factors [2]. ASDs are characterized by a wide range of cognitive, emotional and neuro-behavioural abnormalities. The main core symptoms are dysfunctions in social interactions and communication skills, restricted interests, irritability, repetitive and stereotypic verbal and non-verbal behaviours [3, 4]. Several biochemical and cellular processes are associated with ASDs: oxidative stress; endoplasmic reticulum stress; decreased methylation capacity; limited production of glutathione; mitochondrial dysfunction; intestinal dysbiosis; increased toxic metal burden; immune dysregulation; immune activation of neuroglial cells [5].

Interestingly, ASD disorders are being recognized as public health problem, as their frequency is dramatically increasing: 23 % reported increase since 2009 and 78 % increase since 2007; until to present rates of 11.3 per 1,000 (one in 88) children aged 8 years in US, according to Center for Disease Control [6]. Current pharmacotherapy is not effective for the treatment of core symptoms of ASDs. Indeed, available drugs target specific symptoms without addressing the basic underlying aetiologies [7, 8]. Although a defined standard approach does not exists, current available ASD therapies can be divided into: behavioural, nutritional, psychotherapeutical and pharmacological approaches [9]. It is noteworthy to consider that pharmacological options only target neuropsychiatric disorders (i.e. irritability, depression, anxiety and obsessive-compulsive behaviours) co-associated with ASDs.

Too often, ASDs are underestimated and affected children are poorly addressed. The lack of a specific biomarker for autism diagnosis makes the pathology very

difficult to be diagnosed. A correct and an early diagnosis, together with a full clarification of cellular and molecular pathways involved in the pathology, is priority need for ASD management.

2 Proteases Involvement in ASDs

A recent study has indicated a key role of several caspases in ASDs [10]. The proteases caspases belong to an evolutionarily conserved structurally-related family of aspartate-specific cystein-dependent proteases. They are well known and characterized in apoptosis-related cellular processes [11]. Moreover, beyond programmed cell death, caspases take important roles in several cellular processes and innate and adaptive immune responses [12, 13]. Since their role in cell differentiation, proliferation, as well as in activation and nuclear reprogramming pathways [14], caspases are often referred as pleiotropic enzymes. These enzymes are mainly classified in two major categories: regulatory, or upstream initiator, caspases and effector (downstream) caspases [15]. This classification is based on their activity mechanisms and discovery. Caspases that show regulatory mechanisms are: Caspase-1, -2, -4, -5, -9, -11, -12. They are formed by large pro-domains containing related homotypic oligomerization motifs. Downstream caspases are: Caspase-3, -6, -7, -14. They are also named executioner or effector caspases and are proteolytic activated and matured by the action of the large prodomain caspases. Indeed, it has been demonstrated that caspases are synthesized as zymogens or inactive pro-enzymes with a prodomain of variable length. The following cleavage at specific aspartate cleavage sites is responsible for their activation [16]. In turn, activated effector caspases are able to cleave a wide range of intracellular substrates, i.e. structural and regulatory proteins, leading to a set of biochemical changes in cell morphology and eventual programmed cell death [17]. Interestingly, due to different molecular stimuli, some regulatory caspases (i.e. caspase-12) can also act as executioner caspases.

In the apoptosis process, caspases are responsible of specific cleavage of a wide variety of substrates implicated in the regulatory and execution phases of programmed cell death. Indeed, the cell death is the final outcome of these proteolytic cascade-caspase mediated. On the other hand, in the non-apoptotic processes, caspases are activated independently of an apoptotic stimuli. Once activated, caspases are responsible of the cleavage of a specific subset of substrates, such as cytokines, kinases, transcription factors and polymerases [18].

Another protease family interesting for ASDs is the matrix metalloproteinase (MMP) family. This group comprises secreted and membrane-bound zinc-endopeptidases. It has long been associated with normal physiological processes (i.e. tissue remodelling and wound healing), as well as with pathological diseases (i.e. inflammatory and neurodegenerative diseases) [19]. These extracellular enzymes take a role in synaptic remodelling. Prolonged MMP activity can contribute to neuronal loss and synaptic dysfunction [20]. In ASDs, deficits in synaptic remodelling and brain plasticity could contribute to the development of the pathology.

3 Caspase Involvement in ASDs

3.1 Inflammatory Caspases

Recent evidences highlight immune abnormalities and altered immune responses in ASD pathogenesis [21]. Moreover, caspases are strictly related to inflammatory responses, that are highly altered in ASDs. The main component of this inflammatory caspase group is the well known caspase-1. This one is the first discovered, identified and the best-characterized inflammatory caspase. Caspase-1 is responsible to mediate the pro-inflammatory cytokine production. It is able to cleave the inactive 31 kDa cytokine pro-IL-1β, generating the active 17 kDa mature form of the potent pro-inflammatory cytokine IL-1β [22]. The auto-proteolysis of a large pro-domain forms the two domains of 20 kDa and 10 kDa, respectively, of caspase-1. This autocatalytic process is mediated by the oligomerization and interaction of the prodomain of the initiator caspase with its adaptor protein. In the inflammatory process, the activation of caspase-1 requires the inflammasome formation. This inflammasome is a multiprotein complex acting as an activating molecular platform; indeed, its major functions are recognizing cytosolic pathogen associated molecular patterns and reacting to these patterns through activation of pro-inflammatory cytokines and thereby initiating inflammation [23]. In the inflammasome molecular complex, another caspase, caspase-5, is brought into close proximity with caspase-1 to facilitate the cross-activation [24]. Hence, the activated caspase-1 is able to cleave the pro-IL-1β. It is noteworthy to consider that pro-inflammatory cytokines could induce some of the behavioural symptoms of autism [25]. In addition, the pro-inflammatory cytokine produced by caspase activation are able to trigger innate immune responses. The IL-1β and IL-18, released by caspase-1, initiates Th1- and Th17-mediated adaptive immune responses [13]. These responses are particularly involved in ASD pathogenesis [21]. Increased activation of both Th1- and Th2- mediated immune responses in peripheral blood mononuclear cells (PBMCs) has been demonstrated in ASD patients [21]. ASD-PBMCs show excessive innate and adaptive immune responses, together with an over-production of IL-1β, resulting in long-term immune alterations [26]. Indeed, caspase-1 gene is over-expressed in these ASD-PBMCs, in this way triggering immune response changes through cytokine production.

Caspase-4 and caspase-5 also belong to the inflammatory caspase subfamily [22]. Both of them are involved in cytokine maturation [10, 27]. Caspase-4 is responsible to product IL-18 through TNF Receptor-Associated Factor 6 interaction [28], leading to NF-kB activation. In autism, it has been demonstrated that caspase-4 and -5 mRNAs are highly increased [10]. These caspases act as inducers of altered immune responses in autistic children.

Caspase-12 is another member of this subfamily [29]. It is localized to the endoplasmic reticulum (ER) and activated by ER stress. The ER stress is a complex molecular mechanism induced by accumulation of unfolded protein aggregates. As consequence, the imbalance in Ca^{2+} homeostasis and/or in the protein-folding machinery triggers activation of caspase-12 [30]. ER stress is related to ASD pathology [31]. ER stress is able to induce a trafficking disorder of synaptic receptors and

lead to their impaired synaptic function and signal transduction [32]. Caspase-12 takes a pivotal role in these molecular events in ASD pathophysiology [10].

3.2 Executioner Caspases

In this caspase subfamily are comprised the proteases involved in apoptotic processes. Caspase-3 and -7 are the main representative enzymes. Through interaction and proteolytic activation mediated by large pro-domains of caspase-8 and -9, caspase-3 and -7 are recruited in the apoptosome complex [16]. Active caspase-3 and -7 cleave several substrates, resulting in the morphological and biochemical hallmarks of apoptosis [33]. Interestingly, latest studies have shown that they could also play physiological roles [34]. In ASDs, activated caspase-3 and -7 are responsible for IL-2 production, which in turn enhances T cell activation and proliferation [10, 35, 36]. In this way, caspase-3 and -7 are responsible for triggering immune response imbalance seen in ASDs. Indeed, it has been demonstrated that both these caspases are highly increased and activated in ASD pathology [10].

4 Matrix Metalloproteases Involvement in ASDs

It has been demonstrated that MMP-9 is abundant in amniotic fluid of ASD samples [37]. These results could indicate that neuroplasticity impairments in ASDs may present during pregnancy. In addition, MMPs are involved in Fragile X syndrome (FXS). This syndrome is the most common known genetic form of intellectual disability and ASDs. MMP-9 is elevated also in a mouse model of X-fragile syndrome (FXS), indicating a dysregulation of many proteins important for synaptic plasticity [38]. Indeed, it has been proposed that minocycline, a potent MMP inhibitor, could be effective in treating FXS patients [39]. Further studies need to be performed in order to clarify the exact involvement of these protease family in ASDs.

5 Conclusions and Future Prospects

Despite many research efforts, currently biomarkers for an exact ASD diagnosis do not exist. Proteases could be an interesting potential target to better define the immune and inflammatory molecular pathways involved in ASD pathophysiology. Novel therapeutic strategy in ASDs could be offered by modulating protease activity.

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Conflict of Interest
The authors confirm that this article content has no conflicts interest.

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Role of Proteases in the Management of Hepatitis C Virus

Vaishali M. Patil, Neeraj Masand, and Satya Prakash Gupta

Abstract The essential role of viral NS3/4A serine protease in the process of HCV replication makes the HCV enzyme the most intensively pursued anti-HCV target for drug development. This is also supported by the successful use of small-molecule inhibitors of viral protease and offers hope that analogues molecules might have similar impact against HCV protease. Here, we focus on the recent progress and development of various classes of HCV NS3/4A protease *viz*. macrocyclic, boronate based, phenylglycine based, sulfonamide-capped, and α-ketoamide based protease inhibitors and indoles, oleanolic acids and acridones derivatives. It is expected that detailed characterization of inhibitor binding may provide useful information for the design of inhibitors with the potential to treat resistant cases.

Keywords Hepatitis C Virus • NS3/4A protease • Macrocyclic inhibitors • Boronate inhibitors • Sulphonamides • α-Ketoamides • Phenylglycine based inhibitors

1 Introduction

Recently, two FDA approved linear protease inhibitors were added to the standard-of-care treatment for hepatitis C infection. The two drugs are boceprevir and telaprevir [1]. These drugs marks advances in the treatment of hepatitis C. For the past

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decades, pegylated-interferon plus ribavirin was the standard treatment for chronic hepatitis C infection. Presently available protease inhibitors have many limitations and toxic effects, high costs, increased pill burden, and many drug interactions. Moreover, because new antiviral drugs directly inhibit hepatitis C virus, viral resistance has become an important issue, essentially precluding use of protease inhibitor mono therapy, and potentially restricting future treatment options for patients who consequently do not achieve sustained virological response (SVR). Protease inhibitors are the first of many antiviral medications that will probably be combined in future interferon-free regimens [2].

The hepatitis C virus (HCV) genome was first identified in 1989 by Houghton and co-workers [3]. Presently HCV is the leading cause of liver failure and hepatocellular carcinoma [2]. HCV is a single stranded linear RNA virus with a genome encoding a polyprotein of about 3,000 amino acids. This large polyprotein is cleaved by cellular and viral proteases into structural and non-structural polypeptides and plays an important role in viral replication within hepatocytes. This crucial step involves the serine protease NS3-4A, a protein composed of the N-terminus of the NS3 protein and a small NS4A protein co-factor [4]. The various functions of NS3-4A includes, polyprotein cleavage and subverts host's innate immune response by preventing phosphorylation, and thus activation, of interferon regulatory factor 3, a key antiviral signaling molecule. It induces expression of interferon β, which leads to the expression of many interferon-stimulated genes and produces antiviral state in infected and surrounding cells. NS3-4A has also shown reduction of intrahepatic production of interferon γ, which may impair hepatic inflammatory response and contribute to viral persistence. Thus inhibition of NS3-4A may block viral replication and potentially restore suppressed interferon pathways. Small peptides derived from the cleavage products of NS3 can competitively inhibit this enzyme [1, 5].

2 Structure of HCV Protease

NS3 protease is a heterodimeric enzyme that belongs to the chymotrypsin super family of enzymes. The overall folding is similar to that of other chymotrypsin-like serine proteases. The X-ray crystal structure reveals a shallow, featureless active site, which is highly solvent exposed [6–9]. The serine protease domain is in its ~180N-terminal amino acids, and the remainder of the protein encompasses an RNA helicase [10]. The protease and helicase domains were found segregated and connected by a single strand and the structural segregation is in line with functional studies, showing that the isolated domains retain their respective catalytic activities (Fig. 1) [11–13]. The protease cofactor NS4A is a relatively small protein with only 54 residues. The first ~20 residues of NS4A are highly hydrophobic and were shown to be involved in membrane anchoring of the NS3/4A complex [6]. The NS4A residues 21–34 were shown to directly interact with NS3 and to be absolutely required for the enhancement of its serine protease activity.

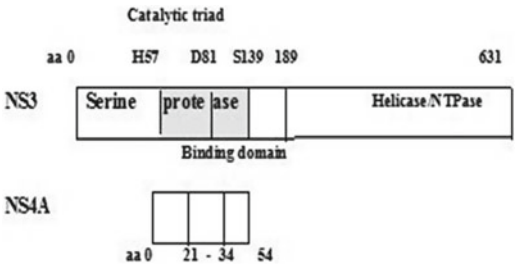


Fig. 1 The Hepatitis C virus NS3 protease

The NS3 protease has two domains and each of which is composed of a β-barrel and two short α-helices. NS4A is bound within the N-terminal domain becoming an integral part of the fold of the protease by forming the seventh strand of an eight-stranded β-barrel. In both the crystal and solution structures of the NS3 proteinase in the absence of NS4A the C-terminal β-barrel adopts essentially the same fold as observed in the complex whereas the N-terminal barrel has a different conformation in the X-ray structure or is disordered in the solution structure. The mechanism of activation of NS3 by NS4A therefore appears to reside in the conformational stabilization of the N-terminal domain of the protease. The catalytic triad of His57, Asp81 and Ser139 is located in a crevice between the two domains. The geometrical arrangement of the catalytic triad is similar to that of other serine proteases. The catalytic triad residues, the oxyanion cavity (residues 135–139) and β-strand E2, forming one side of the specificity pocket, have the same relative spatial orientation as in other chymotrypsin-like serine proteases. A characteristic feature of NS3 furthermore is the presence of a Zn ion that is coordinated tetrahedrally by Cys97, Cys99, Cys145 and His149 at a site located opposite to the active site [14–16]. The Zn ion is believed to play a structural role, as its removal was shown to lead to unfolding and precipitation of the protein [14]. It is interesting to note that the metal binding site is located at one end of a long loop that connects the two domains of NS3. Other chymotrypsin-like proteases contain disulfide bridges in a topologically similar position. Since disulfide bridges are not stable in the reducing intracellular environment the metal binding site of NS3 has possibly evolved as a reductant-stable, structural homolog of the disulfide bridges usually found in extracellular serine proteases.

The NS3-dependent cleavage sites of the HCV polyprotein have the consensus sequence D/E-X-X-X-XCys/Thr↓Ser/Ala-X-X-L/W/Y, with cleavage occurring after cysteine or threonine [17, 18]. As shown by substrate specificity studies [19–22], cysteine clearly is the preferred P1 residue and is found in 3 out of the 4 cleavage sites. The only exception is the intramolecular NS3/4A junction that harbors a threonine residue in this position. Other conserved features are a negatively charged residue in the P6 position, an alanine or a serine in P1′ and a hydrophobic residue in the P4′ position. Analysis of the cleavage kinetics of different peptide substrates has shown that the minimum length required for a synthetic substrate is a decamer spanning from P6 to P4′ and incorporating all of these conserved features. This rather unusual requirement for large peptide substrates can be rationalized through the structural analysis of the substrate binding site as revealed by the X-ray crystal

structure. In fact, the NS3 protease lacks all major loops that in other enzymes of this family make up the P2 and P3 binding sites, leading to a remarkably shallow and solvent exposed substrate binding region. The binding energy for the substrate is derived from a series of weak interactions that are distributed along an extended contact surface and that involve all of the evolutionary conserved features of the cleavage site sequences. This situation makes the design of potent, small molecule inhibitors a quite challenging task.

3 Role of NS3/4A Protease

For HCV replication, both the protease and the helicase activities are important. The C-terminal half of NS2 with the N-terminal protease domain of NS3 forms a catalytically active protease to cleave the NS2/3 junction [23]. Heterodimerization of serine protease with its cofactor NS4A significantly enhances proteolytic processing efficiency. This protease is responsible for the cleavage of the viral polyprotein at four sites *viz*. NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B [24, 25].

The co-factor NS4A of HCV along with the amino-terminal domain of the NS3 protein forms a heterodimeric protease. It cleaves the viral polyprotein into single functional proteins. The substrate specificity of NS3-4A protease is very different from that of host cell serine proteases [26, 27]. The flat or shallow shape of substrate-binding region is the major difficulty in the design of selective inhibitors [28]. Various groups were successful in the designing of highly selective inhibitors of this enzyme.

The HCV NS3/4A serine protease is not only involved in viral polyprotein processing, but also efficiently blocks the retinoic acid-inducible gen I (RIG-I) and Toll-like receptor (TLR)-3 signaling pathways and contributes to virus persistence by enabling HCV to escape the interferon antiviral response [29, 30]. NS3/4A protease inhibition can prevent Cardif and/or Toll/interleukin-1 receptor domain-containing adaptor inducing interferon (TRIF) inactivation during HCV infection, thereby maintaining the innate immune response [31]. Thus, differences in NS3/4A protease catalytic efficiency could be related to viral pathogenicity. Both its essential role in the HCV life cycle and its ability to counteract host innate immunity make the NS3/4A protease an ideal target for the development of new anti-HCV agents and control of the disease [32].

4 Mechanism of Protease Inhibition

The rate of cure of HCV infection has been greatly increased with the advances in the new family of drugs called NS3/4A protease inhibitors. HCV's single strand of RNA contains the code for a single large polyprotein that must be cleaved into a handful of smaller proteins, some of which (structural proteins) make up the new

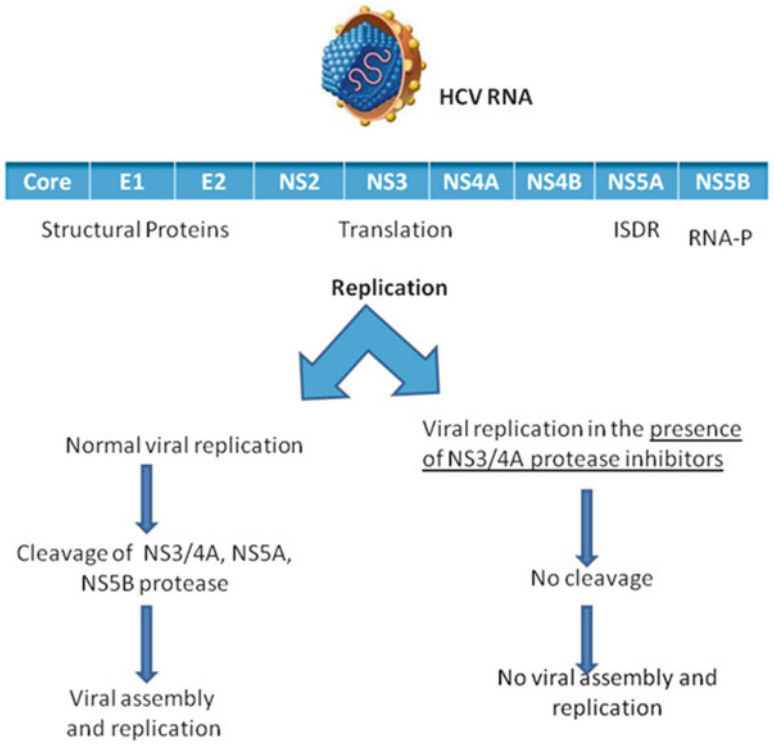


Fig. 2 Mechanism of HCV protease inhibition

virion and the rest of which (nonstructural proteins) mediate the process. Some of the enzymes (proteases) that cleave the polyprotein are supplied by the host cell; others are nonstructural proteins contained in the polyprotein [33, 34]. For optimal proteolytic activity of NS3, its interaction with NS4A is essential [35]. Compounds that disrupt or affect the outcome of the protein–protein interaction between NS3 and NS4A (for example, by induction of a conformational change) could potentially interfere with viral polyprotein processing by the NS3 protease and thus viral replication. Indeed, several peptides derived from NS4A, which compete with NS4A for binding to NS3, inhibited the protease activity *in vitro* in low micromolar values [36]. Furthermore, NS4A binding appears necessary for NS3 to associate with the ER membrane, and may affect the stability of the NS3 protein [37], or internal cleavage of NS3 [38] and NS2–NS3 processing [39]. The mechanism of HCV protease inhibition is shown in Fig. 2 [40].

NS3/4A protease inhibitors rely on the principle of end-product inhibition, in which the cleavage product of the protease (a peptide) acts to inhibit the enzyme activity; this is why they are called peptidomimetics. The active site of the NS3/4A protease is a shallow groove composed of three highly conserved amino acid residues, which may explain why protease inhibitors display high antiviral efficacy but

pose a low barrier to the development of resistance [40]. Some features remains to be explored, the multiple functional outcomes assigned to the NS4A–NS3 interaction suggest that it might offer another avenue to intervene NS3 function.

5 Clinical Success of Protease Inhibitors

The development of small molecule inhibitors of NS3/4A protease as antiviral agents has been intensively pursued as a viable strategy to eradicate HCV infection. Protease inhibitors are very efficacious in lowering viral loads to undetectable levels. However, the development of effective inhibitors is a daunting task [41]. Inhibitors of the NS3/4A protease have been the most extensively studied direct antivirals to date. According to their chemical structures, HCV protease inhibitors are classified as non-covalent (linear peptidic inhibitors and macrocyclic inhibitors) or covalent (linear peptidic inhibitors and macrocyclic inhibitors). Some of the clinically successful molecules are represented in Fig. 3.

BILN 2061 (ciluprevir) was the first specific NS3/4A protease inhibitor to enter clinical trials. In phase I studies it has shown promising results, but further trials were halted due to cardiac toxicity in rhesus monkeys [42]. Ciluprevir has opened the door to future trials with NS3/4A protease inhibitors. Currently, a number of

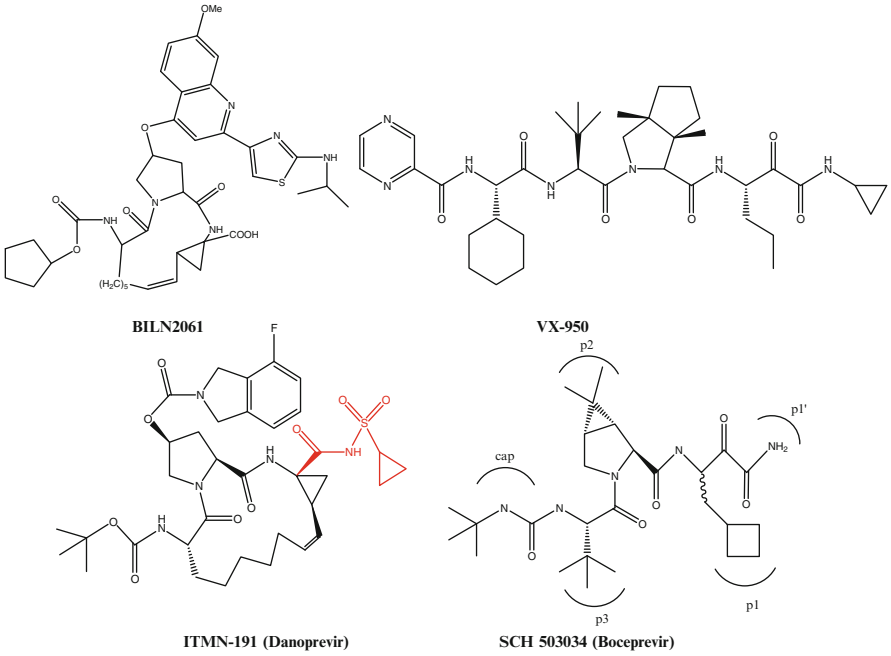


Fig. 3 Protease inhibitors in clinical phases

compounds from Boehringer Ingelheim, Vertex, ViroBay and Abbott/Enanta are in phase I clinical trials; Merck, Roche, Achillion, and Boehringer Ingelheim in phase II; and, Merck and Vertex in phase III [43] (Chen and Njoroge, 2009). Two agents, the peptidomimetic inhibitors telaprevir and boceprevir, are most advanced.

Telaprevir was developed by Vertex Pharmaceuticals using a structure-based drug design approach and it is a covalent reversible peptidomimetic inhibitor of the HCV NS3-4A protease [44]. The end-point of phase II trials was sustained virological response (SVR) [45]. A carboxamide-based HCV NS3-4A oral protease inhibitor, Boceprevir (SCH-503034) has been reported by Schering-Plough [46]. It is an orally active compound and entered phase IIb trials. Further InterMune developed ITMN-191 (R7227), a non-coalent macrocyclic acylsulphonamide-containing oral inhibitor [47] and found to be effective against several HCV genotypes [48]. Similarly TMC435350, a new macrocyclic acylsulphonamide-containing reversible HCV NS3-4A protease inhibitor [49]. MK-7009 is again a macrocyclic inhibitor of HCV NS3-4A. The SCH6 is a α-ketoamide derived, substrate-based inhibitor of HCV NS3-4A protease [50] and its consideration for therapeutic use is on hold. Achillion and Gilead developed ACH-806, an NS4A antagonist [51] but further development has been discontinued due to abnormal elevations in serum creatinine levels due to nephrotoxicity [52].

VX-950 is yet another peptidomimetic inhibitor of the viral protease VX-950 (Fig. 3) that was discovered using a structure based drug design approach. VX-950 has a different mode of binding to the viral protease than BILN 2061. The latter is a noncovalent, reversible serine protease inhibitor, whereas VX-950 forms a covalent but reversible complex with the HCV NS3-4A serine protease in a slow process (slow-on). This allows formation of the covalent bond between the HCV protease and VX-950. Once formed, the covalent complex dissociates slowly back to the free enzyme and inhibitor with a half-life of about 1 h [53].

This may provide a sustained inhibitory antiviral effect. Also in contrast to BILN-2061, VX-950 retains good activity against genotype 2 HCV proteases [54]. The major in vitro resistance mutation against VX-950 is a substitution of Ala 156 to Ser. Replicons carrying this mutation remained sensitive to BILN 2061 [55]. Reciprocally, the dominant in vitro resistance mutations against BILN 2061, substitutions of Asp 168 with Ala (D168A) or Val (D168V), remained fully sensitive to VX-950 [55, 56]. In a 14-day phase 1b trial of VX-950 in genotype 1 HCV-infected patients, a 4.4-log10 median reduction in the plasma viral load was observed in a group of patients dosed with 750 mg of VX-950 every 8 h. In some patients the virus became undetectable at day 14 of dosing [57]. The protease inhibitor SCH 503034 (Shering Plough) (Fig. 3) is an orally active compound. In particular mutation A156T resulted in a >100-fold resistance to the compound [58–60]. The reduced fitness of the resistant variant may suggest that development of high-level resistance in the clinical setting may be slow. As will be likely the case with several other compounds, combination therapy with IFN-α should also greatly reduce the potential emergence of resistance. In an ongoing phase II study of genotype-I infected patients (non interferon responders), 400 mg of SCH 503034 thrice daily as single therapy resulted in a mean HCV RNA reduction of 2 log10 [46].

Recent studies suggest that NS3-mediated cleavage of host factors may abrogate cellular response to IFN-α. Blockage of NS3 protease activity is therefore expected to inhibit HCV replication by both direct suppression of viral protein production as well as by restoring host responsiveness to IFN-α [61, 62]. The macrocyclic peptidomimetic BILN-2061 (Boehringer Ingelheim) a non-covalent inhibitor of NS3-4A protease inhibitor, was the first HCV protease inhibitor to enter clinical trials. The compound resulted, within hours after administration, in a rapid decline of HCV RNA levels. Activity was most pronounced in HCV genotype-1 infected patients [63]. The inhibition was transient as viral (RNA) levels returned to base-line upon cessation of therapy. However, the hope is that longer treatment with protease inhibitors, either alone or in combination with other compounds, may result in (markedly) higher rates of sustained response than with the current standard therapy.

6 HCV NS3/4A Protease Inhibitors

On the basis of mechanism of inhibition protease inhibitors can be classified as non-covalent product-based inhibitors (ex. BILN-2061), and covalent reversible inhibitors (ex. Telaprevir, Boceprevir) [64]. The α-keto amide, telaprevir inhibits NS3-4A protease binding to the enzyme active site in two steps. In the first transient phase it binds to the enzyme non covalently followed by a slow rearrangement to a covalently bound complex. Slow dissociation of the stable complex gives a relatively long half-life.

6.1 Synthetic Analogs

6.1.1 Macrocyclic Inhibitors

In recent years a large number of protease inhibitors have been developed for the treatment of various diseases and most of them are peptidic in nature [65]. A series of phenylalanine-based macrocyclic HCV inhibitors (**1a-b**, Fig. 4) are reported by Chen et al. [66]. The 17-membered macrocycles were designed as peptidomimetic of a P2-P3 dipeptide moiety. The C-terminal carboxylic acids and amides were found as potent protease inhibitors with *Ki* values between 0.066 and 0.12 μM, and the *tert*-butyl esters were less active. The X-ray structure revealed adoption of a 'donut-shaped conformation' by the macrocyclic ring around the methyl group of Ala156. As compared to noncyclic analogs, the macrocyclic inhibitors were few folds more potent.

A P3 aza-peptide analog of a potent macrocyclic tripeptide inhibitor (**2**, Fig. 5) closely related to BILN 2061 was synthesized [67]. It was found to be >2 orders of magnitude less active than the parent macrocycle in both isolated enzyme HCV NS3-4A and HCV subgenomic replicon assays. Further molecular models of these

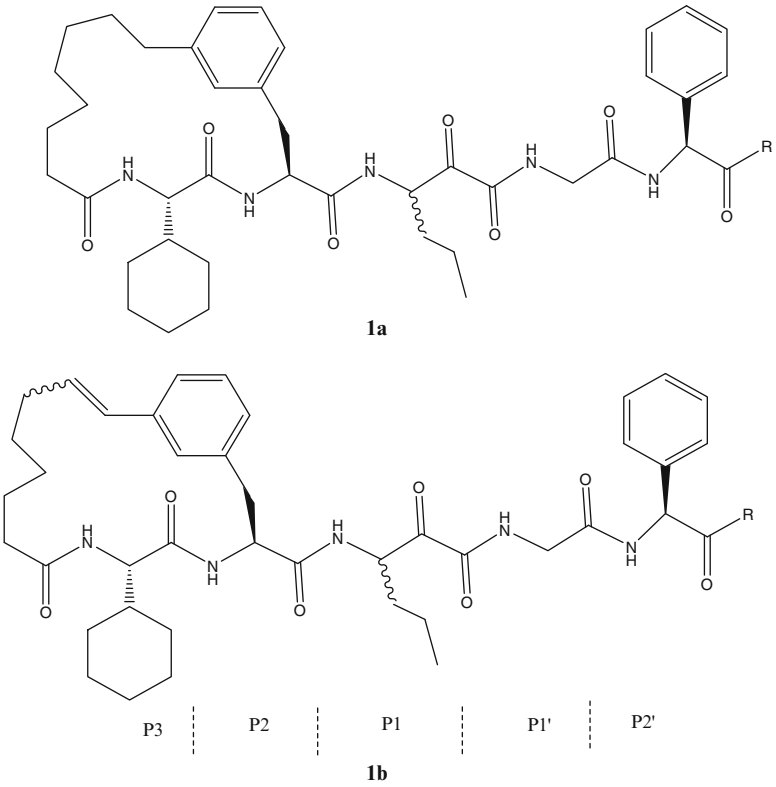


Fig. 4 Phenylalanine-based macrocyclic HCV inhibitors

structures indicate a D-like configuration of the P3 aza-residue and adoption of H-bond stabilized conformation which is different from that necessary for tight binding to the active site of HCV NS3 protease. Scientists at IRBM and Merck Research Laboratory reported novel P2–P4 macrocyclic inhibitors (**3** and **4**, Fig. 5) of HCV protease and from the same structural class, MK-7009 is having a cyclopropylacylsulfonamide in P1. It is currently in clinical investigation [68–71].

Pompei et al. [72] explored the replacement of the P3 carbamate with a succinamide motif to develop P2–P4 macrocyclic inhibitors of HCV protease. The depeptidization strategy resulted in analogs **5a** and **5b** (Fig. 6) having sub-nanomolar enzyme potencies and good cell based activities (K_i = 0.61 nM, K_i = 0.76 nM, EC_{50} = 11 nM and EC_{50} = 12 nM 10 % FCS, respectively). The results concluded use of succinamide motif as a suitable peptidomimetic approach having favorable effects on plasma exposure and liver levels of the corresponding derivatives due to modulation of their physicochemical properties. The 7-methoxy-2-phenylquinoline analog **5c** was identified as a P2 heterocyclic moiety having K_i = 0.60 nM, EC_{50} = 37 nM. Further studies are underway to expand the combinations of P2 heterocyclic cores with substituents on the succinamide nitrogen optimized at the S4 pocket of the protease.

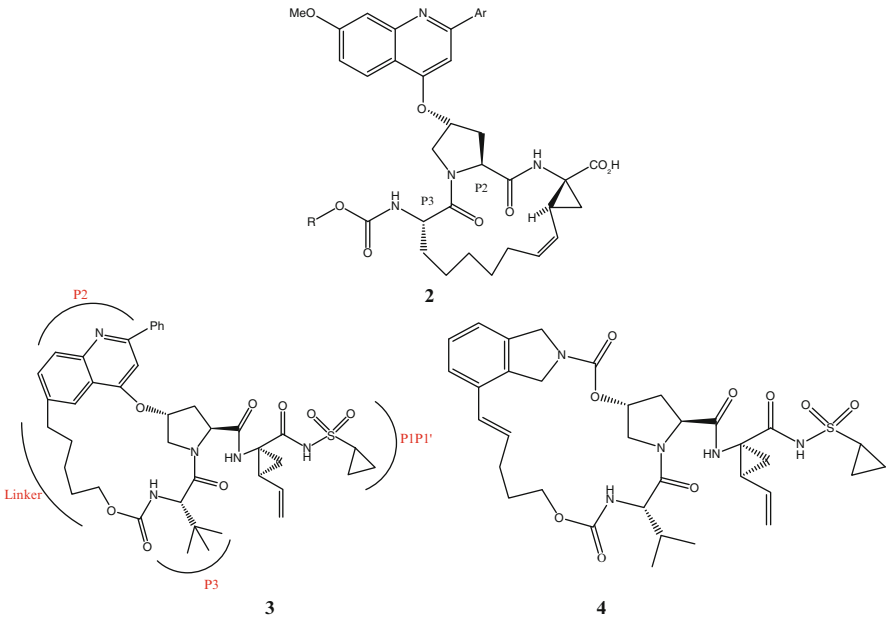


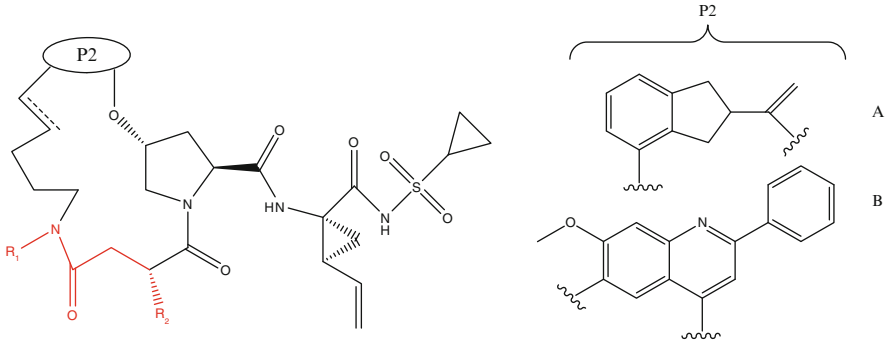
Fig. 5 Structures of macrocyclic protease inhibitors

Nair et al. [73] reported synthesis and SAR studies on the extension of P3 unit of Boceprevir (SCH 503034) having amides and lactams. Compound **6** (Fig. 6) having 4,4-dimethyl lactam was identified as a new P4 cap unit with improved potency (K_i = 15 nM, EC_{90} = 70 nM) and pharmacokinetic properties (Rat AUC (PO) = 3.52 μM h) as compared to Boceprevir.

The alternative carboxylic acid bioisosteres having phosphonates/phosphinates as potential acid replacements were evaluated with an aim to identify novel P2–P4 macrocyclic inhibitors of NS3-4A [70]. The P2–P4 macrocycles bearing acidic phosphorous group position and phosphonic/phosphinic moiety at the P1carboxylic acid functionality (Fig. 7) were evaluated. The methyl-phosphinates (**7**) was characterized as most active analogs with K_i = 0.0024 μM, EC_{50} = 0.50 μM (FCS) and pK_a = 3.1. Venkatraman and Njoroge [74] have described various novel approaches used in the design of macrocyclic inhibitors linking the P2–P4 residues and P1-P3 groups. The binding interactions were elaborated in correlation to the crystal structure as shown in Fig. 8.

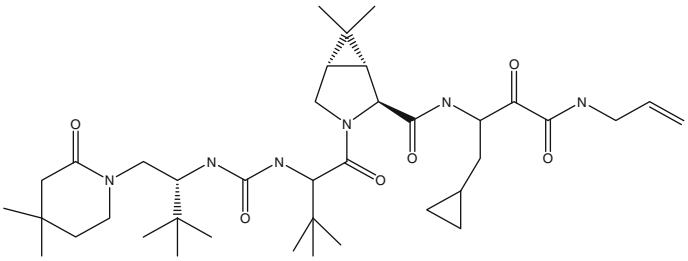
6.1.2 Boronate Based Inhibitors

Scientists at Roche and DuPont designed a novel series of α-amino boronic acid (ester) containing inhibitors of HCV NS3/4A protease with assumption that *C*-termini boronic acid or ester could serve as "serine trap" by forming reversible, covalent



5a-b

Compd	P2	Linker	R_1	R_2	K_i (nM)	EC_{50} (nM)
5a	A	Satd	*i*Pr	c-Hex	0.61	11
5b	A	Satd	H	c-Hex	0.76	12
5c	B	Satd	*i*Pr	c-Hex	0.60	37



K_i = 15nM, EC_{90} =0.070μM, HNE/HCV = 870

6

Fig. 6 Some P2–P4 macrocyclic inhibitors

interaction of the boron with the catalytic serine hydroxyl group. Some examples of the peptidyl and non-peptidyl based inhibitors are as shown in Fig. 9 [75].

In compounds VX-950 and SCH-503034, the most common feature is the presence of α-ketoamide moiety at the P1 position and it acts as an active-site serine trap, essential for inhibitory activity. A series of novel α-amino acid boronates (**14a**, Fig. 10) has been designed and it was incorporated in several acyclic templates at the P1 position. The SAR studies have concluded influence of ring size, chirality, and substitution patterns. The X-ray structure of boronate inhibitor **14b** complexed with HCV NS3 protease explains the mechanism of inhibition *i.e.* by trapping Ser139 in the enzyme active site [76].

The peptide framework and P1 carboxylic acid region was optimized to reduce the toxic effects. The P1 carboxylic acid is replaced with many different groups like tetrazole, acylcyanamide, acysulfonamide, phosphonate [70, 77–79]. Among them, cyclopropyl acylsulfonamide appears to be the preferred replacement for the P1

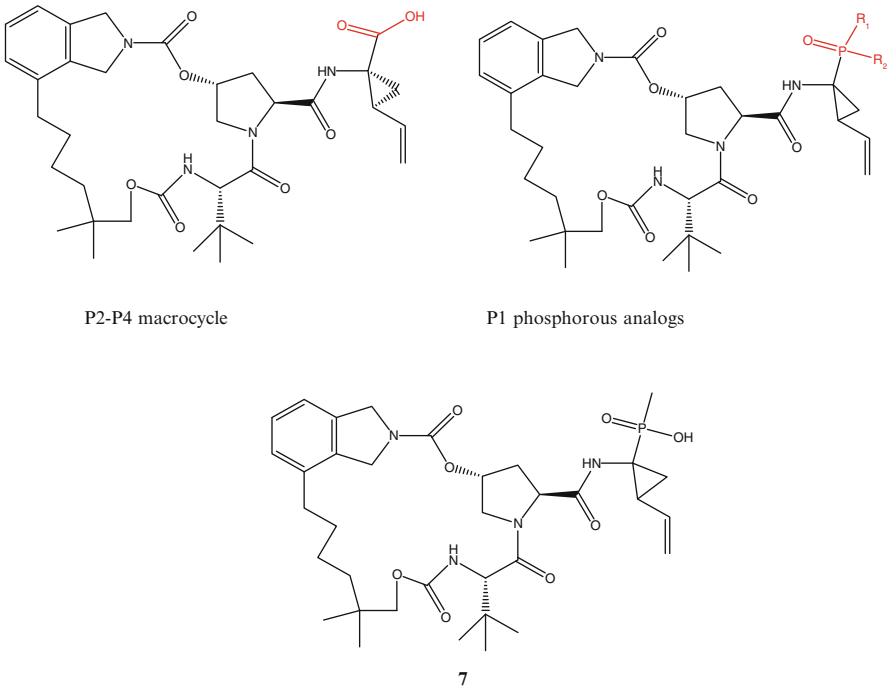


Fig. 7 Targeted phosphorous acid analogues

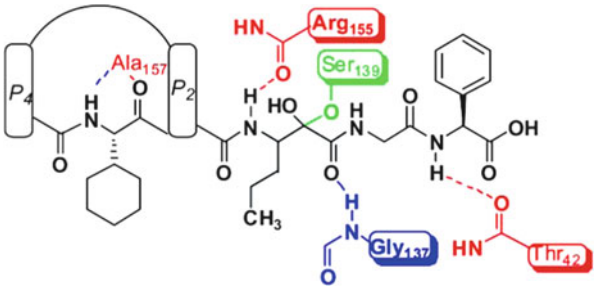


Fig. 8 Binding interactions of macrocyclic inhibitor, compound **8**

carboxylic acid [80, 81] and some examples of this approach includes discovery of number of clinical candidates including ITMN-191 (danoprevir) [82], TMC-435350 (medivir) [83] and MK-7009 (vaniprevir) [84], currently in advanced clinical trials.

Li et al. [85] reported series of unoptimized P1–P3 and P2–P4 marocyclic inhibitors having comparable activity to that of danoprevir. The benzoxaborole moiety was further optimized to rebalance the physicochemical properties and to improve their membrane absorption, potency and bioavailability.

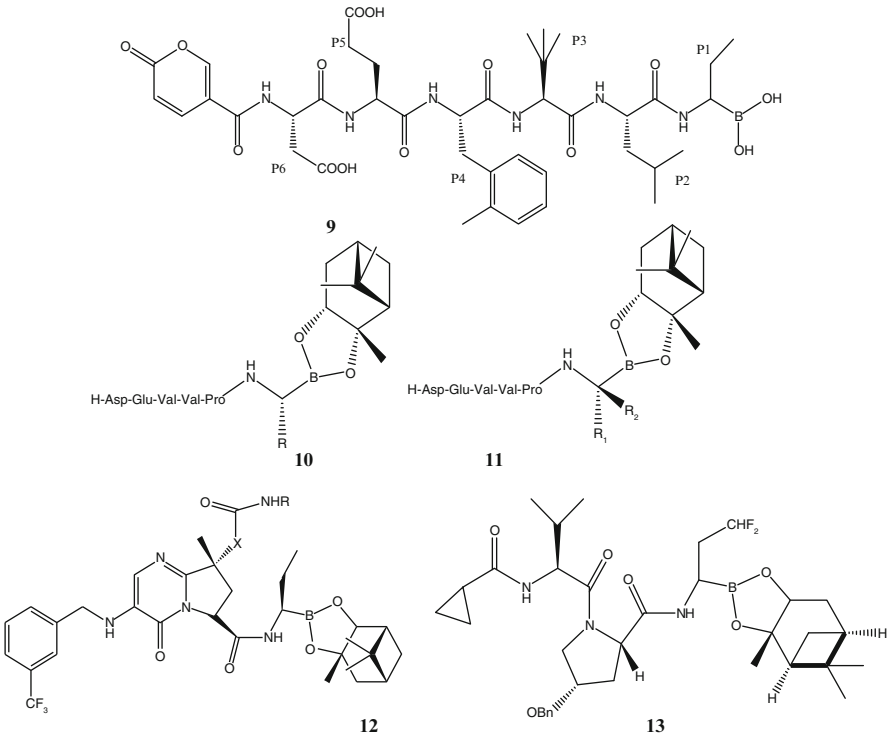


Fig. 9 Peptidyl boronate based inhibitors

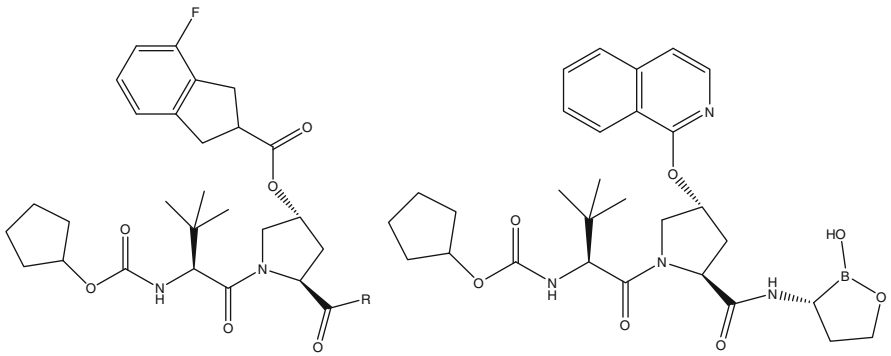


Fig. 10 α-Amino acid boronates

The macrocyclic inhibitors have shown cardiac problems in treated animals and this prompted study of novel acyclic inhibitors. It includes BI-201335 and BMS-650032 [86–88]. In 2011, Li et al. evaluated novel acyclic P4-benzoxaborole-based HCV NS3 protease inhibitors having various linkers and substitutions around the

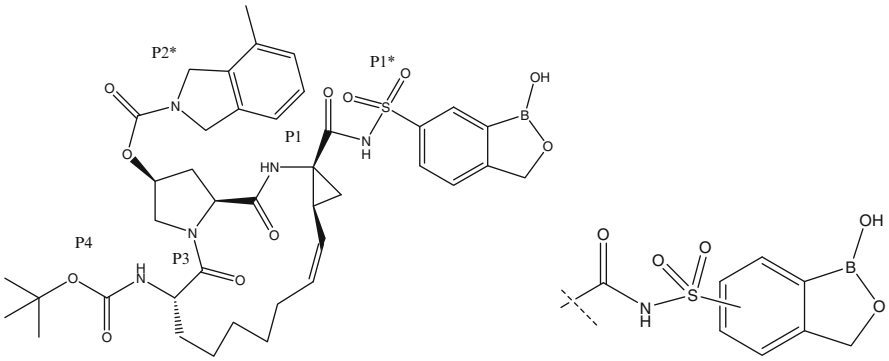


Fig. 11 Hypothetical P1–P3 macrocyclic inhibitor in which an acylsulfamoyl benzoxaborole is used to replace the cyclopropyl acylsulfonamide in danoprevir

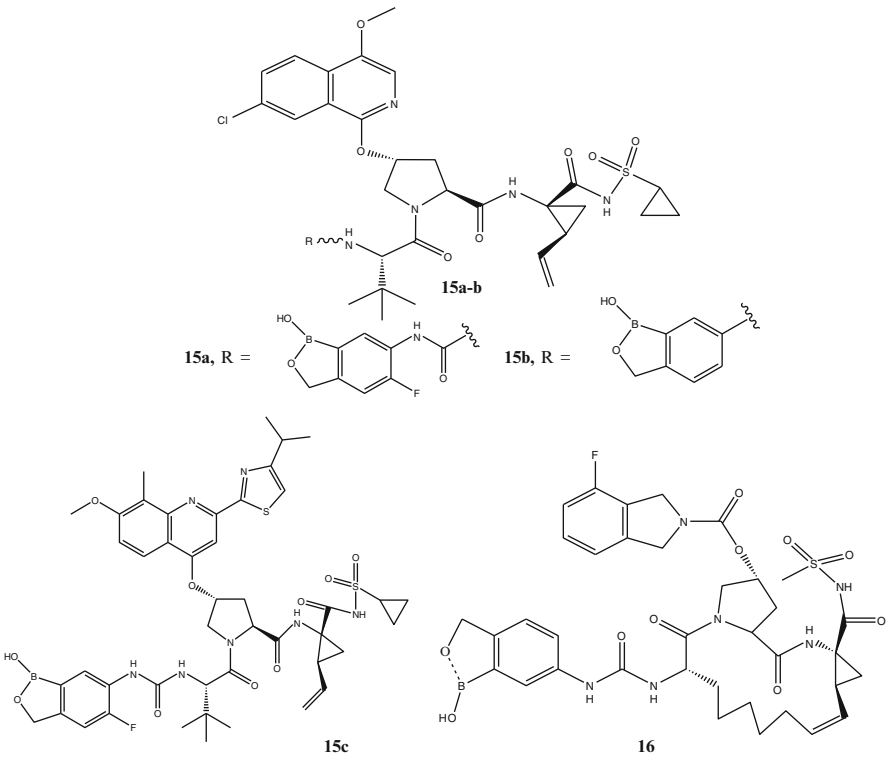


Fig. 12 Structures of acyclic P4-benzoxaborole based HCV NS3 protease inhibitors

benzoxaborole moiety and the P2* groups (Fig. 11) [89]. The reported series ((Fig. 12) has shown nanomolar potencies in enzymatic and cell-based replicon assays. Compound **15a**, the fluoro-containing acyclic inhibitor has cell-based

potency comparable to the macrocyclic compound. Further compound **15c** exhibited three- to fourfold improved potency compared to **15b**. In rat PK model the oral bioavailability was low in most of the compounds while the linker-truncated compound **15b** has shown better in vitro and in vivo absorption and better bioavailability. It suggests further reduction of molecular weight and PSA to improve drug-like properties.

Ding et al. [90] designed and reported a series of P4-benzoxaborole-substituted macrocyclic HCV protease inhibitors (**16**, Fig. 12) with aim to retain potency against resistant enzymes. Docking studies to the active site of enzyme suggested suitable orientation and linkage can potentially interact with active site polar amino acid residues like Ser122, Arg123, Arg155 and Asp168. Various substitutions on the benzoxaborole ring system and P2* groups has shown impact on cellular replicon potencies. The high polar surface area (PSA) has shown limitations towards oral absorption and bioavailability.

6.1.3 P2 Phenylglycine Based Inhibitors

In HCV NS3 protease inhibitors, Phenylglycine (Phg) has been reported as an alternative P2 moiety, invoking an advantageous resistance profile [78, 91]. Structural moieties as vinyl-ACCA in P1 were not found beneficial and thus suggest an alternative binding mode. Further chemical modifications are allowed by the aromaticity of Phg. The modeling and inhibition experiments have suggested the possibility of retention of necessary conformation by Phg-based inhibitors (*i.e.* while binding to A156T and D168V mutated forms of the protease). To optimize the Phg, a vinyl was introduced at the 3-position along with an alkenylic P1′ elongation (**17**, Fig. 13). The alkenylic P1′ extensions proved to be beneficial and the vinylic substitution has shown positive effect for D-Phg based inhibitors [92]. Further macrocyclization was attempted to validate the accuracy of docked model as well to probe the bioactive conformation of Phg-based inhibitors [93]. A series of tripeptide P2 Phg-based HCV NS3 protease inhibitors (**18**, Fig. 13) with a macrocycle spanning P1′ and P2 is reported. The ring closed inhibitors were found to have lower potency than their acyclic counterparts and thus suggests beneficial interactions with H57 were disrupted after macrocyclization.

Molecular modeling and inhibitory potencies of tetrapeptide protease inhibitors (**19**, Fig. 13) of HCV NS3 proposed phenylglycine as a new promising P2 residue. The results suggest that phenylglycine might be capable of interacting with the NS3 (protease-helicase/NTPase) in ways not possible for the common P2 proline-based inhibitors. Thus, a series of tripeptides, both linear and macrocyclic, based on p-hydroxy-phenylglycine in the P2 position were prepared and their inhibitory effect determined. When the *p*-hydroxy group was replaced by methoxy, isoquinoline-, or quinolinyloxy functions, inhibitors with improved potencies were obtained. The P2 phenylglycine-based inhibitors were further optimized by C-terminal extension to acyl sulfonamides and by P1–P3 cyclization, which gave products with inhibition constants in the nanomolar range (~75 nM) [78].

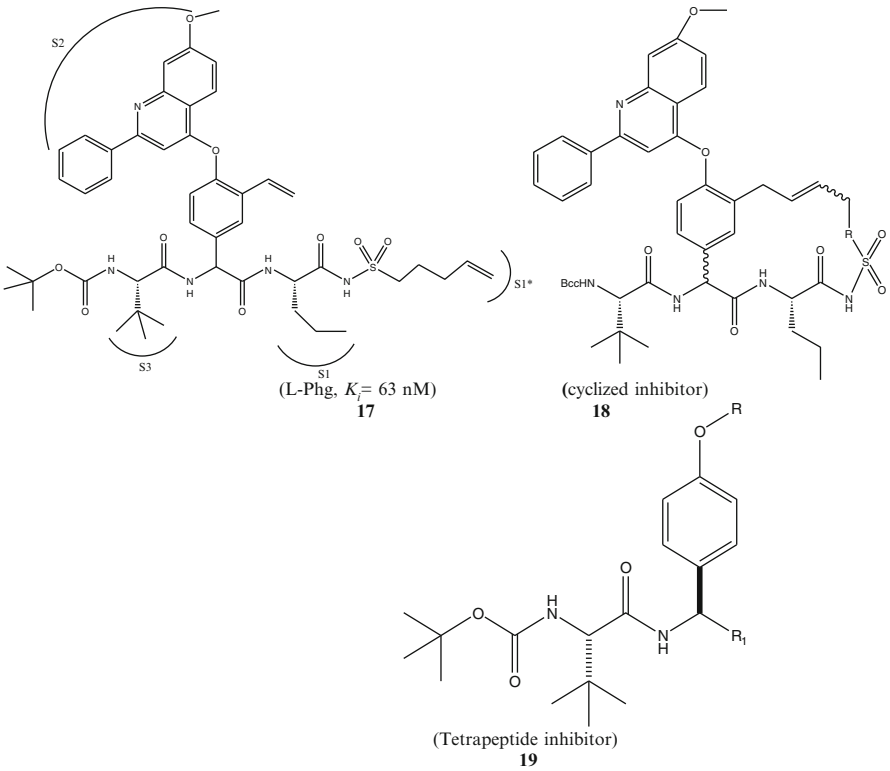


Fig. 13 Structures of P2 phenylglycine based inhibitors

6.1.4 α-Ketoamide Based Inhibitors

This is a class of potent and slow inhibitors of the HCV NS3 protease and show interaction with the enzyme by forming a reaction intermediate into a tight binding complex. Narjes et al. [94] explored that the α-keto moiety at *C*-termini serves as the serine trap. The compound **20** is shown in Fig. 14. The *C*-termini carbonyl group is attacked from the *si*-side by the catalytic serine and forms a covalent bond with the inhibitor via the Oγ, resulting into a tetrahedral intermediate. The two carboxyl oxygens point into the oxyanion hole by forming two hydrogen bonds with Ser139 N and Gly137 N, respectively.

6.1.5 P_3 Sulfonamide-Capped Inhibitors

Some P3-capped sulfonamide series of inhibitors (**21a-b, 22** of Fig. 14) having more potent activity than SCH-503034 were synthesized and evaluated [95]. It was observed than replacement of *tert*-butyl urea group with valine-derived P_3 capping group yields compound **21a** having improved cellular potency. Further replacement

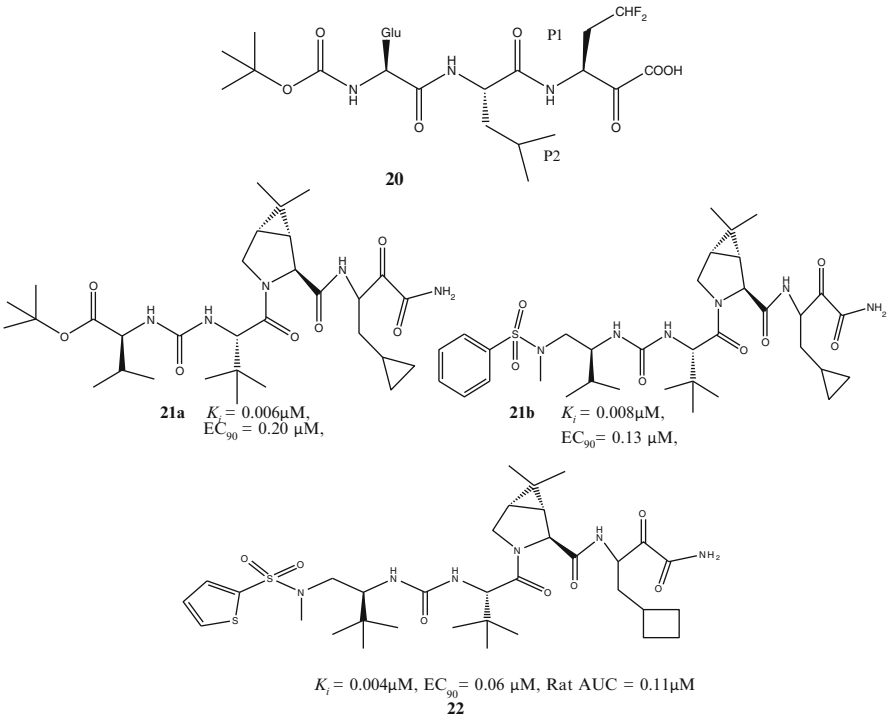


Fig. 14 Structures α-ketoamide based and sulfonamide-capped inhibitors

of P_3 valine cap with modified sulfonamide moiety gave compound **21b**. SAR studies concluded identification of sulfonamide and sulfonyl urea series of inhibitors with improved cellular potency compared to SCH 503034 and *tert*-butyl glycine was identified as an optimal P_4 group. Compounds of type **22**, primary ketoamide inhibitors have shown EC_{50} <0.1 μM.

6.1.6 Indole Derivatives

A new series of protease inhibitors (**23**, Fig. 15) were developed based on the molecular modeling of inhibitor bound to NS3/4A protease structures. Compounds having $R_1 = R_2 = OMe$, $R_3 = Phenyl$ and $R_1 = CF_3$, $R_2 = OMe$, $R_3 = Phenyl$ have shown high fit values and docking scores. The same analogues proved to be most potent during *in vitro* protease binding assay [96].

Further designing of indole based peptidomimetic replacements for the *N*-terminal amino acid of potent tripeptide "serine trap" inhibitor (**24**, Fig. 15) is reported [97]. Heterocyclic moieties were added to retain the double hydrogen bonding interaction with Ala157; orient a P2-P1 amino acid sequence as an active site "anchor"; direct pendant functionality to engage the P3/S3 side chain interaction; and to function as a bridge to auxiliary binding regions, such as those accessed by the N-terminal capping group in the peptide series.

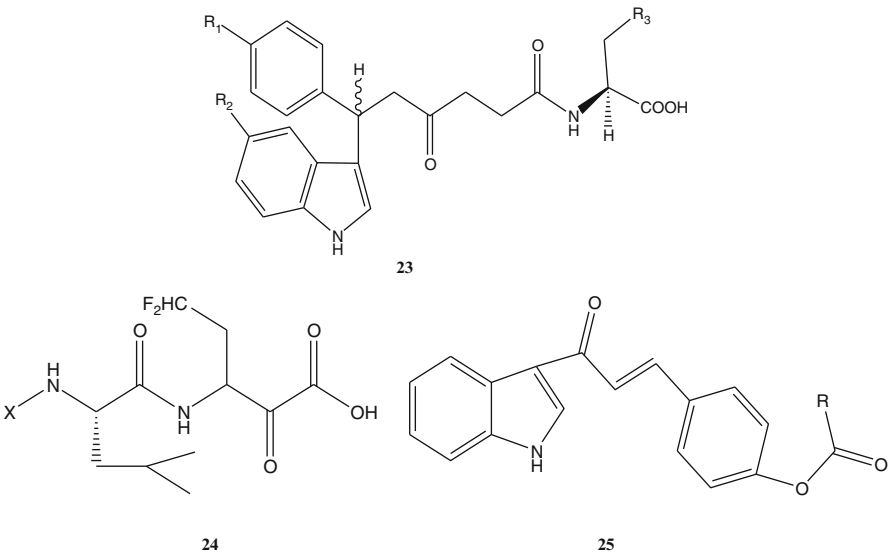


Fig. 15 Examples of indole type of protease inhibitors

Ismail et al. [98] performed molecular modeling studies of a series of indole derivatives (**25**, Fig. 15) and evaluated in vitro the compounds with significant high simulation docking score and fit values. Compounds having R = -NH-CH(COOH) $CH_2C_6H_5$; and -NH-CH(COOH)CH_2-4-OH-C_6H_5) demonstrated potent HCV NS3 protease inhibitors with IC_{50} values of 9 and 12 μg/ml, respectively. They concluded promising characteristics of this class for further development of anti-HCV agents.

6.1.7 Acridone Derivatives

Starting from hit compounds **26a-b**, a large series of acridones and acridone-fragment derivatives (Fig. 16) were synthesized and evaluated for their ability to inhibit the HCV replication in Huh-5-2 cells.

Some new selective anti-HCV compounds were identified revealing that the acridone skeleton, when properly functionalized, represents a suitable scaffold for obtaining potential anti-HCV agents. Structure features responsible for the HCV replication inhibition are a methoxy, ethoxy, or isobutyloxy group as a substituent, a 4-arylpiperazine as a side chain with 1-(2-pyridinyl)-, 1-(4-pyridinyl)-, and 1-(1,3-thiazol-2-yl)piperazine which confer the best activity, a RIV methyl group, and an amino group or hydrogen atom as an RVI substituent. Searching for their molecular target, we have identified the thiazolpiperazinyl derivative **23** which inhibited, albeit with low potency, the HCV NS3 helicase. Studies are in progress to gain insight into the precise mechanism of action of **27**, which would ultimately provide a structural basis for the discovery of improved anti-NS3 helicase acridones. For all the other selective acridones identified in this study, which did not recognize

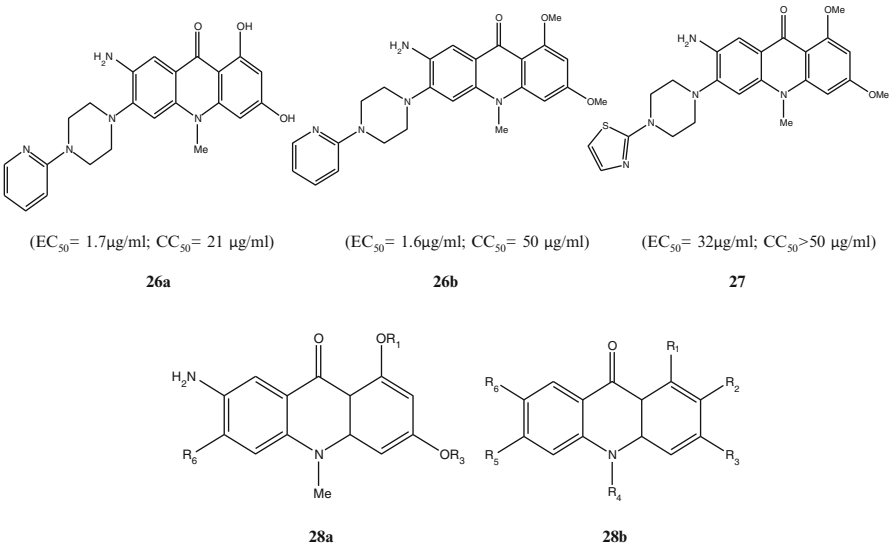


Fig. 16 Examples of some acridone derivatives

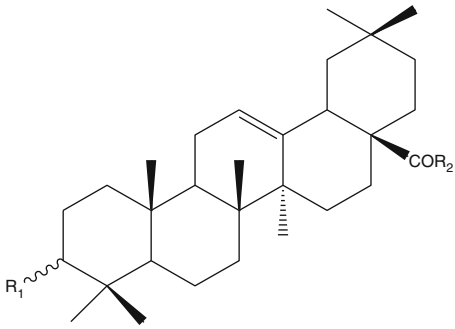
the NS3 helicase, the anti-HCV replicon activity could be due to either a different unknown mechanism of action or a polypharmacological effect. The latter interpretation is in line with what has been previously reported by Bastow29 for other antiviral acridones, for which more than one biochemical target has been identified [99].

The reported selective HCV inhibitory profile of acridone derivatives (**28a–b**, Fig. 16) stimulated Manfroni et al. [100] to evaluate a series of acridone and acridone-fragment derivatives. The SAR studies concluded potential of acridone skeleton towards HCV inhibitory activity.

6.1.8 Oleanolic Acid Derivatives

Ma et al. [101] reported series of oleanolic acid derivatives (represented by structure 29, Fig. 17) as P2–P4 macrocyclic inhibitors of hepatitis C viral protease as well as for their cytotoxic and apoptosis-inducing effects on Hep G2 cells. The amino derivatives (R_1 = -NH-R) showed potent cytotoxicity and the β-amino isomer showed strong apoptosis-inducing effect and exhibited more distinct cytotoxicity than the α-isomer. The cytotoxicity of hemiesters and hemiamides varied as the chain lengths varied. The oxalic and malonic hemiesters showed weaker cytotoxicity than oleanolic acid (R_1 = β-OH, R_2 = OH), while those with longer chain lengths at R_1 showed higher cytotoxicity. All the hemiesters and hemiamides showed high activity against HCV protease. It concluded that addition of amino-group is contributing towards improving cytotoxicity and the introduction of acidic group increases the inhibition on HCV protease may be useful for further design and synthesis of triterpene derivatives as drug candidates for liver diseases.

Fig. 17 Structure of oleanolic acid derivative



29

6.2 Natural Analogs

Duan et al. [102] reported purification and evaluation of three polyphenol compounds (**30a–c**, Fig. 18) from the EtOAc extract of fraction of the Traditional Chinese medicine (TCM) *Galla Chinese*. The isolated compounds **30a** (1,2,6-tri-*O*-*galloyl*-β-D-glucose), **30b** (1,2,3,6-tetra-*O*-galloy-β-D-glucose), and **30c** (1,2,3,4,6-penta-*O*-galloyl- β-D-glucose) has shown inhibition of HCV NS3 protease with IC_{50} of 1.89, 0.75, and 1.60 μM, respectively.

Li et al. [103] reported application of molecular dynamics simulations combined with MM-PBSA (Molecular Mechanics and Poisson–Boltzmann Surface Area) to predict the binding mode of the polyphenol inhibitors (Fig. 18) in the binding pocket of the HCV NS3 serine protease for which the ligand–protein crystal structure is not available. The most favorable geometry from molecular docking had a binding free energy about 3 and 6 kcal/mol more favorable. The correlation of the calculated and experimental binding affinities of all five polyphenol compounds is satisfactory ($r^2 = 0.92$). The most favorable binding mode has shown that two galloyl residues at 3 and 4 positions of the glucopyranose ring of the inhibitors interact with Ser139, Gly137, Ala157, and Asp81 by hydrogen bond interaction and with Ala156 and His57 by hydrophobic interaction and are found as essential for the NS3 inhibitory activities.

7 Conclusions

The HCV NS3/4A serine protease is the focus of many academic and pharmaceutical industry researches. The available crystal structures of the NS3/4A enzyme have allowed innovative lead generation and subsequent lead optimization for NS3/4A inhibitors. The challenges of designing potent inhibitors of the viral protease have been solved, as highlighted by the success of teleprevir and boceprevir. Still it is too early to determine whether all these efforts will eventually yield promising drug candidates. For the emerging small-molecule HCV inhibitors, viral resistance will

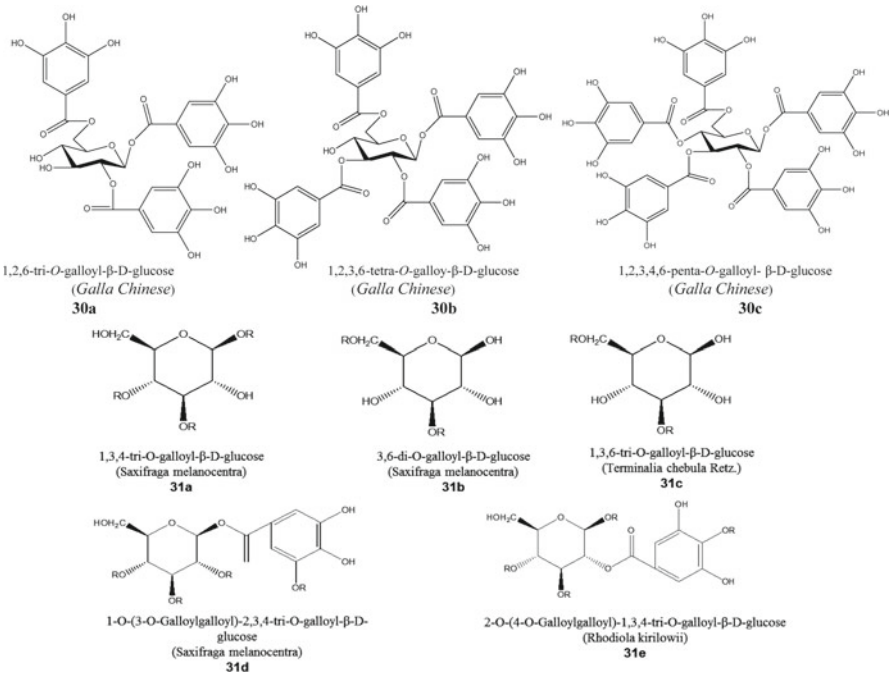


Fig. 18 Structures and resources of polyphenols from natural sources

likely be a big problem. Thus, combination therapy of different drugs with different targets/mechanisms will be necessary to effectively inhibit HCV replication. It is also hoped that a detailed characterization of how the resistance mutations that affect NS3 inhibitor binding may provide useful information for the design of inhibitors with the potential to treat resistant viruses that may arise during chronic HCV infection.

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Role of Proteases in Inflammatory Lung Diseases

Kaustav Chakraborty and Arindam Bhattacharyya

Abstract Proteases are enzymes that have the capacity to hydrolyze peptide bonds and degrade other proteins. Proteases can promote inflammation by regulating expression and activity of different pro-inflammatory cytokines, chemokines and other immune components in the lung compartment. They are categorized in three major subcategories: serine proteases, metalloproteases and cysteine proteases especially in case of lung diseases. Neutrophil-derived serine proteases (NSPs), metalloproteases and some mast cell-derived proteases are mainly focused here. Their modes of actions are different in different diseases for e.g. NE induces the release of IL-8 from lung epithelial cells through a MyD88/IRAK/TRAF-6-dependent pathway and also through EGFR MAPK pathway. NSPs contribute to immune regulation during inflammation through the cleavage and activation of specific cellular receptors. MMPs can also influence the progression of various inflammatory processes and there are many non-matrix substrates for MMPs, such as chemokines, growth factors and receptors. During lung inflammation interplay between NE and MMP is an important significant phenomenon. They have been evaluated as therapeutic targets in several inflammatory lung diseases. Here we review the role of proteases in various lung inflammatory diseases with emphasis on their mode of action and contribution to immune regulation during inflammation.

Keywords Serine Protease • Metalloprotease • Inflammation • Inflammatory lung diseases • Pro-inflammatory cytokines • Chemokines • Neutrophil

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1 Introduction

Numerous environmental pathogens, particulate matters, allergens and harmless antigens are present in the air we breathe. Although airways are the main port of entry for pathogens and allergens during inhalation of the inspired air (10,000 L per day in humans) [1], the lung is one of the most challenged organs of the body. For this reason, air-breathing animals have developed several defense mechanisms in this compartment [2, 3].

Inflammation, a host defense mechanism, is an immediate response of the body to tissue injury caused by harmful stimuli, such as pathogens, damaged cells or chemicals [4]. Lung inflammation is a broad term which covers various acute and chronic inflammatory diseases such as Acute Lung Injury (ALI), Acute Respiratory Distress Syndrome (ARDS), Emphysema, Airway hyper-reactivity (AHR) or Asthma, Allergic Asthma or Chronic eosinophilic inflammation, Chronic Obstructive Pulmonary Disease (COPD), Fibrotic lung disease, Pulmonary edema, Tuberculosis, Pneumococcal infection etc. This complex and dynamic process is characterized by an innate immune response, which involves a coordinated expression of inflammatory cytokines and implication of various cell types particularly immune cells aimed at clearing the pathogenic agent, damaged cells etc. [5]. Modulation of expression and activity of these inflammatory cytokines and other immune components are regulated by various proteases in the lung compartment. Here we present the information relative to some relevant proteases which have roles in different inflammatory lung diseases.

Proteases are enzymes that have the ability to hydrolyze peptide bonds and degrade other proteins. According to the active groups of their catalytic center they are categorized and in case of lung disease, basically three major protease group serine proteases, cysteine proteases, and the matrix metalloproteases (MMPs) are studied [6]. There is another group the ADAM (a disintegrin and metalloprotease) family of proteases, which has an emerging role in mucin production and cytokine processing [7]. A number of studies have elucidated the role of proteases in human diseases such as cancer, thrombotic and inflammatory disorders [8, 9]. Various inflammatory cells, such as neutrophils, mast cells, macrophages, and lymphocytes are the major source of proteases within the lung [10–12]. Other cells, including epithelial, endothelial, and fibroblasts, also synthesize proteases [13, 14]. Serine proteases, including Neutrophil Elastase (NE), Cathepsin G (CG), and Proteinase 3 (PR 3), are packaged in primary granules within neutrophils [15]. Some of the metalloproteases, MMP-8 and MMP-9, are also packaged into specific and gelatinase granules, respectively, in the neutrophil [16].

2 Proteases in Lung and Their Mode of Action

In the lung, proteases function either intracellularly or extracellularly after cellular activation. Proteases that may be present in the respiratory tract and activate PARs include the endogenous enzymes mast cell tryptase (activates PAR2), trypsin

(PAR1, PAR2 and PAR4), chymase (PAR1) and cathepsin G (PAR4), as well as exogenous enzymes such as Der p1 (PAR2) that are inhaled. However, these and other enzymes within the respiratory tract may also inactivate or disarm various PARs by cleaving them at other sites that remove the tethered ligand sequence [17]. Proteases have crucial role in chemotaxis of all of the various inflammatory cell types to the lung. MMP-9 and serine proteases increase eosinophil chemotaxis, and MMP-12 is responsible for eosinophil and macrophage accumulation [18, 19]. However, maximum work has focused on neutrophil chemoattraction to the lung by interleukin 8 (IL-8) and leukotriene B4 to study the effect of proteases on cell migration in inflammatory lung diseases.

3 Serine Proteases

3.1 Neutrophil-Derived Serine Proteases (NSPs)

Neutrophils are essential for host defense against invading pathogens. They are the first inflammatory cell lines to enter tissue during inflammation [20]. They engulf and degrade microorganisms using an array of weapons that include reactive oxygen species, antimicrobial peptides, and proteases such as Cathepsin G, Neutrophil Elastase and Proteinase 3. After release, these proteases also contribute to the extracellular killing of microorganisms, and regulate non-infectious inflammatory processes by activating specific receptors and modulating the levels of cytokines [21]. In addition to their involvement in pathogen destruction and the regulation of proinflammatory processes, NSPs are also involved in a variety of inflammatory human conditions, including chronic lung diseases (chronic obstructive pulmonary disease, cystic fibrosis, acute lung injury, and acute respiratory distress syndrome) [22–25]. In these disorders, accumulation and activation of neutrophils in the airways result in excessive secretion of active NSPs, thus causing lung matrix destruction and inflammation.

3.2 Neutrophil Elastase (NE)

NE is a serine-protease of the chymotrypsin family stored in primary (azurophilic) granules of PMNs along with proteinase-3 and cathepsin G, two other neutrophil serine-proteases. Intracellular stored NE comes in action when azurophilic granules are incorporated to the phagosome [26]. However, *in vitro* stimulation of PMNs with physiological relevant pro-inflammatory stimuli induces either transfer of NE to the plasma membrane (membrane-bound NE associated to by proteoglycans) [27, 28], or a secretion in the extracellular space specially in case of pulmonary chronic (CF and COPD) [29, 30] or acute lung injury [22], where high efflux of PMNs in the alveolar space increase the release of NE from necrotic PMNs. Functions of NE are not only concerned with degrading bacteria [31–33] and

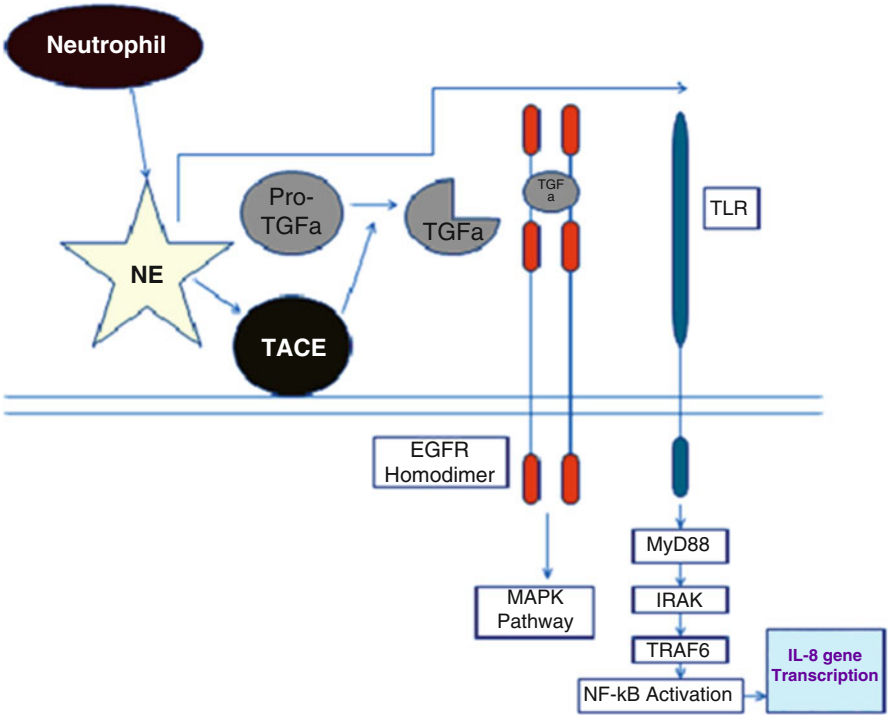

Fig. 1 Mechanism of neutrophil elastase (NE) induced-release of IL-8 from lung epithelial cells. Following its release from the azurophilic granules in response to pathogenic/pathologic insult, NE activates TNFa converting enzyme (TACE), which in turn cleaves proTGFa (pro-transforming growth factor a) to generate soluble TGFa as a ligand for the epidermal growth factor receptor (EGFR). EGFR co-localizes with toll-like receptor-4 (TLR4) and a signal transduction cascade is initiated via myeloid differentiation factor 88 (MyD88 or Mal), IL-1 receptor-associated kinases (IRAKs), tumor necrosis factor receptor-associated factor 6 (TRAF6), transforming growth factor-beta-activated kinase 1 (TAK1) and the IkB kinases (IKKs), leading to a degradation of inhibitor of NF-kB (IkB) proteins, activation of nuclear factor-kB (NF-kB) and increased IL-8 gene transcription

extracellular matrix molecules, they also operate on various bioactive molecules including chemokines, cytokines, growth factors and cell surface receptors [34–36], thus the "deleterious" concept of NE has changed towards a multifunctional molecule able to regulate inflammatory process and immune responses. Indeed, extracellular NE (free, chromatin-bound or membrane-bound) participates in: (1) direct killing of bacteria [31–33]; (2) processing and release of chemokines, cytokines and growth factors [34, 35], (3) modulation of immune cell activity through interaction with cell surface receptors [36, 37], (4) mucus secretion [38].

Proteases can also modulate cytokine activity and release from immune cells through mechanisms independent of cytokine receptors. For example, it has been shown that NE induces the release of IL-8 from lung epithelial cells [39–42] through a MyD88/IRAK/TRAF-6-dependent pathway [42] that also involves TLR4 [39] and also through EGFR MAPK pathway (Fig. 1) [43]. How NE activates TLR4 is

unknown but liberation of proteolytic fragments from host targets able to recognize PRR could be possible as described for TLR2 [44]. Serine proteases such as NE can induce IL-8 expression by bronchial epithelial cells (Fig. 1) and leukotriene B4 expression by macrophages [42, 45]. NE appears to be the most important regulatory factor present in the cystic fibrosis (CF) lung responsible for IL-8 expression because inhibition of NE activity in CF bronchoalveolar lavage fluid (BALF) almost completely blocks IL-8 message in bronchial epithelium [40]. It has been shown that NE generally act at least in part via an IL-1 receptor-associated kinase-1/myeloid differentiation factor-88/nuclear factor-κB-dependent pathway in bronchial epithelial cells; this can be inhibited by a dominant negative variant of myeloid differentiation factor-88 [42]. This gives new therapeutic approaches targeted at inhibiting the NE-activated intracellular pathways rather than NE itself. It is quite clear that expression of IL-8 and leukotriene B4 are responsible for neutrophil migration to the lung, and given the high neutrophil and NE burden present in the CF lung this has lead to the "vicious cycle" hypothesis whereby NE is the main player behind IL-8 production and neutrophil influx into the CF lung. From further experiments it has been found that an initial inflammatory event can stimulate further inflammation i.e., epithelial cell injury in mice leads to secretion of the murine homolog of IL-8, which in turn binds to an adhesive component of the extracellular matrix, syndecan-1 [46]. MMP-7 cleaves this syndecan-1-murine IL-8 complex and this is crucial for attracting neutrophils to the damaged epithelial surface (Fig. 2).

3.3 Proteinase-3 (PR-3)

Proteinase-3 (PR-3) is a serine protease that cleaves TNF into membrane-associated TNF and soluble TNF form [47]. It has been hypothesized that PR-3-mediated TNF processing may be an important mechanism in inflammatory lung diseases [48]. PR-3 is able to degrade extracellular matrix, and its potential involvement in pulmonary inflammatory disease has been demonstrated by the induction of emphysema in hamsters following intratracheal instillation [49]. PR-3 is also enhanced in the sputum of cystic fibrosis patients, and correlates with disease severity [50]. The main sources of PR-3 are neutrophils and it has been hypothesized that there would be an increased contribution of PR-3 to TNF processing in diseases with abundant alveolar neutrophils such as usual interstitial pneumonia (UIP) [48].

NSPs contribute to immune regulation also through the cleavage and activation of specific cellular receptors. NE, PR3, and CG can process the N-terminal extracellular domains of protease-activated receptors (PARs), which are a subfamily of related G-protein-coupled receptors [51, 52]. These receptors are ubiquitously expressed in various tissues and cells and, more especially, in platelets and endothelial cells. Processing of PAR extracellular domains occurs through exposure of a tethered ligand that allows the auto-activation of the receptor and subsequent activation of an intracellular signaling cascade via phospholipase C [51, 52]. Four PARs have been identified so far; three of them, PAR-1, PAR-3, and PAR-4, can be activated by thrombin. Apart from thrombin, CG released from activated neutrophils

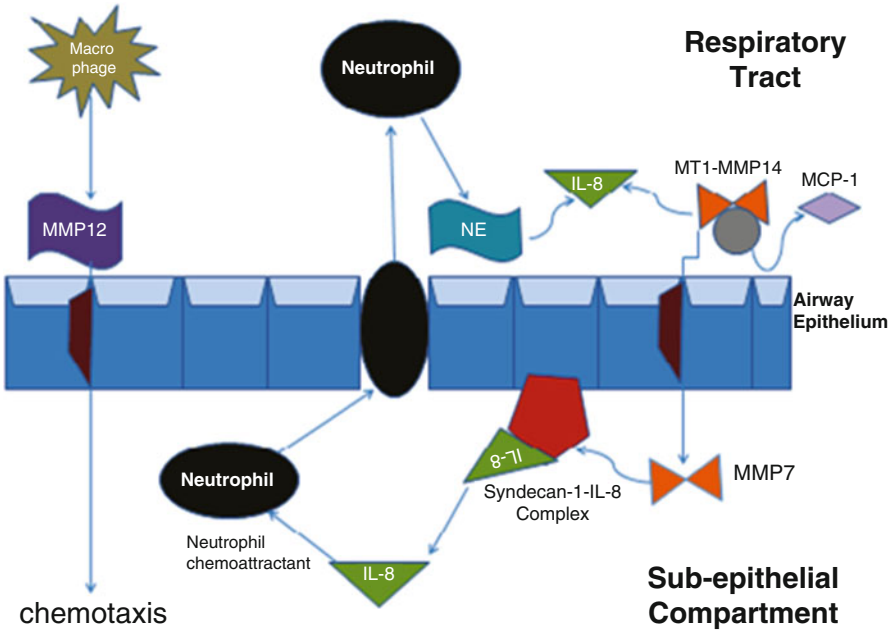

Fig. 2 Interaction of NE and MMPs during inflammatory responses in the respiratory tract. Neutrophil Elastase (NE) induces interleukin-8 (IL-8) synthesis, resulting in chemoattraction of neutrophils to the respiratory tract. MT1-matrix metalloprotease (MMP) 14 processes IL-8 and monocyte chemoattractant protein 1 (MCP-1), and MMP-7 cleaves the syndecan-1-IL-8 complex to generate active IL-8, which in turn acts as a neutrophil chemoattractant. MMP-12 released from macrophages also helps in chemoattraction of different inflammatory cell

can also activate PAR-4 at the surface of platelets and initiate their aggregation [53]. All three NSPs cleave PAR-1, which impairs their activation by thrombin [54]. Serine proteases cleave the amino acids at a specific site of the extracellular N-terminus of the molecule to expose a new N-terminal ligand domain that binds to another site on the same molecule, thereby activating the receptor. The amino acid sequence of each cleavage site is specific for the particular PAR, and mAb(monoclonal Antibody) assays for the protein and PCR assays for mRNA are available [55]. Activation of PAR2 results in the production and secretion of IL-8 and chemokine (C-Cmotif) ligand 2 [56, 57].

3.4 Human Airway Trypsin-Like Protease (HAT)

HAT belongs to the family of Type II Transmembrane Serine Proteases (TTSP) [58]. Members of this family present a short intracellular domain connected to a single-pass transmembrane domain followed by a large extracellular domain containing a highly variable stem region and a C-terminal serine-protease domain of the

chymotrypsin (S1) fold [58]. It is preferentially expressed in human bronchial and tracheal respiratory tract [59, 60], particularly in ciliated cells [61]. A soluble form after proteolytic cleavage is also found in the sputum of patients with chronic airways diseases [59]. Concerning the lung compartment, *in vitro* experiments have revealed various HAT activity such as: (1) fibrinogenolytic activity in lung airway anticoagulation processes [62]; (2) proteolytic inactivation of urokinase-type plasminogen activator receptor (uPAR) in lung epithelial cells [63]; (3) proteolytic activation of the hemagglutinin antigen of influenza virus leading to multicycle replication and propagation of influenza virus *in vitro* [64, 65]; (4) stimulation of lung fibroblast proliferation through a PAR-2-dependent MEK-MAPK mediated pathway [66]; (5) increase of intracellular Ca^{2+} concentration in bronchial cells through PAR-2-dependent mechanisms [67].

4 Metalloproteases

4.1 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are zinc-dependent neutral endopeptidases that form a family of extracellular matrix proteolytic enzymes. They are primarily responsible for the degradation of extracellular matrix components during the remodeling processes essential for normal tissue growth and repair. In the lung, inappropriate expression and excessive activity of several MMPs, including MMP-12, have been implicated in the tissue-destructive processes associated with chronic lung diseases, including COPD and asthma [68–73]. It is well established that MMP-12 has definite role in the pathogenesis of COPD [74]. Patients suffering from COPD have increased secretion and activity of MMPs, especially MMP-2 and MMP-9, been identified in inflammatory cells and tissues isolated from those patients [75]. In our previous study, we found increased expression of MMP-2 in case of cadmium induced lung inflammation in mice model [76]. Kundu *et al.* (2009) performed SDS-PAGE and gelatine zymography to find out the expression of MMP-2 from lung cell extracts at different time points after the induction of cadmium chloride and found there was increased expression of MMP-2 even after use of ibuprofen, a non-steroidal anti-inflammatory drug (Fig. 3). Expression and activities of MMP-2 and MMP-9 are increased in case of Influenza virus infection both *in vivo* and *in vitro*. It has been demonstrated that H3N2 virus infection induces expression of MMP-2 and MMP-9 in murine lungs *in vivo* and alveolar epithelial cells *in vitro* [77]. Gelatinases (including MMP-2 and MMP-9) are zinc-dependent endopeptidases, degrade major components of the basement membrane such as gelatin and collagen IV, and exert deleterious effects on the epithelium and endothelium in the thin alveolar-capillary barrier [78].

A number of studies reveal that MMPs can also influence the progression of various inflammatory processes and there are many non-matrix substrates for MMPs, such as chemokines, growth factors and receptors [79]. MMP-14 is a protease who

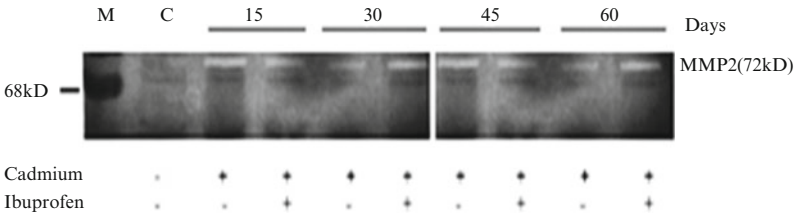

Fig. 3 Increased expression of MMP-2 in cadmium induced lung inflammation by SDS-PAGE and Gelatin zymography. Detection of matrix metalloproteinase-2 (MMP-2) expression at different time points after the induction of Cadmium chloride (5 mg/kg body weight). Lung cell extracts were prepared at days of 15, 30, 45 and 60 from normal (N), Cadmium treated and Cadmium plus Ibuprofen treated (three individual animals per dose) mice. The zymography was developed and stained. The picture shows increased expression of MMP-2(72 kDa), which was not inhibited by Ibuprofen. Gel is representative of three comparable experiments indicate $p<0.05$ with respect to the control

does the processing of IL-8 by removing a pentapeptide from the N-terminus of the protein, resulting in a more biologically active form. MMP-9 also process IL-8 to a form 20-fold more active as a chemoattractant [80, 81]. They process monocyte chemoattractant proteins (MCPs) to produce antagonists of chemokine receptors, indicating a role for MMP-14 in dampening inflammation [82]. Another potent pro-inflammatory cytokine IL-1β that requires proteolytic processing before activation not only by caspase-1 but also several MMPs, including MMP-2, MMP-3 and MMP-9. Interestingly, MMP-3 can degrade the mature IL-1beta cytokine, suggesting potentially dual roles for MMPs in either stimulating or inhibiting IL-1beta effects [83]. The mechanism by which MMPs control inflammation is the regulation of chemokine gradients and that includes both the immobilization of chemokines to the components of extracellular matrix and the generation of chemotactic concentration gradients which provide indications for leukocyte migration. Thus, MMPs can indirectly control influx of inflammatory cells by cleaving proteins in the pericellular environment that bind chemokines. One well established example of this mechanism is MMP7-dependent shedding of syndecan-1 in acute lung injury [46]. In response to lung injury, both CXCL1 (KC) and MMP7 are induced, and MMP7 sheds syndecan-1, a ubiquitous heparan sulfate proteoglycan, that releases the CXCL1-syndecan-1 complex to generate a chemokine gradient. MMP7-null mice that lack this shedding are unable to create a CXCL1 gradient, and thus, neutrophils fail to efflux into the alveolar space and instead remain in the perivascular space [84].

There is also interplay between NE and MMPs in inflammation. NE activates MMP-9 directly and indirectly by inactivating TIMP-1, the naturally occurring inhibitor of MMP-9 [85, 86]. Furthermore, NE may activate MMP-2 through a mechanism that requires MMP-14 expression [87]. Therefore, a variety of proteases liberated from neutrophils or actively expressed on epithelial cells may interact with each other, thereby perpetuating a cycle of inflammation. If we consider CF lung, there is high concentration of NE due to the increased neutrophil burden present in the CF lung and surplus the levels of MMP-14-processed monocyte chemoattractant

protein antagonists. This is supported by evidence showing significant IL-8 levels present in CF bronchoalveolar lavage fluid [40].

4.2 ADAM (A Disintegrin and Metalloprotease)

ADAMs are a family of type I transmembrane proteins belonging to the adamalysin subfamily of metalloproteinases [88]. Members of this family present a metalloprotease domain and a domain of interaction with integrin (disintegrin domain) [89], indicating that ADAMs are both proteases and adhesion molecules. At least 40 ADAMs has been described so far, 25 of which are expressed in humans [90, 91]. ADAMs family has been implicated in the control of membrane fusion, cytokine and growth factor shedding, and cell migration, as well as processes such as muscle development, fertilization, and cell fate determination.

Pathologies such as inflammation and cancer also involve ADAMs family members. ADAMs has been related to lung pathological processes such as cancer [90], asthma [92, 93] and idiopathic pulmonary fibrosis [94]. ADAMs can also modulate cell responses to various signals by acting as cell surface sheddase on membrane associated cytokines, apoptosis ligands, growth factors and receptors. In particular, sheddase activity has important physiological consequences. One of the most documented example is through the production of active tumor necrosis factor-α (TNF-α), a potent inducer of innate inflammatory responses. TNF-α is a homotrimeric transmembrane protein of 26 kDa able to induce inflammatory and cytotoxic effects after cell-to-cell contact [95]. ADAM-17 (also known as TNF-α convertase enzyme or TACE) cleaves transmembrane TNF-α to release the 17 kDa active soluble form of TNF-α [96]. Importantly, ADAMs are also implicated in the shedding of most of the EGFR ligands (EGF, transforming growth factor (TGF)-a, heparin binding (HB)-EGF, betacellulin, epiregulin and amphiregulin (AR)) [89]. It has been reported that Adam17 knock-out mice presented developmental defects resembling those in animals lacking TGF-a, HB-EGF, AR, or the EGFR [97–99]. In addition, sheddase activity on EGFR ligands has important physiological consequences for mucus production. Recently, it has been confirmed that endothelial ADAM10 and ADAM17 are both required for microvascular permeability [100]. To investigate this phenomenon Dreymueller *et al.* (2012) did LPS treatment on HMVEC-L (Human Microvascular Endothelial Cells) and found there is release of soluble JAM-A by LPS-challenged HMVEC-L. Release of soluble JAM-A was 1.3-fold enhanced by 4 h of LPS stimulation, further increasing to 2.3-fold by stimulation for 24 h and was completely inhibited by GW280264X-treatment, capable of blocking tumor necrosis factor-alpha-converting enzyme (TACE) and the closely related disintegrin-like metalloproteinase 10 (ADAM10). The knockdown of ADAM10 or ADAM17 by shRNA indicated the involvement of ADAM17 and to a lesser extent of ADAM10 in JAM-A release. They further examined whether ADAM10/17 activity might influence transendothelial migration of neutrophils. The inhibitor

GW280264X reduced transmigration in response to the neutrophil-attracting cytokine IL-8 by 70 %. Silencing or knock-out of either ADAM10 or ADAM17 alone was sufficient to abrogate transmigration in response to IL-8. Thus, endothelial ADAM10 and ADAM17 are both required for microvascular permeability and for IL-8-mediated transmigration of neutrophils *in vitro*.

4.3 TACE (TNF-α Converting Enzyme)

It has been reported that TACE mediates a critical step in the development of post-transplantation lung injury [101]. Goto *et al.* (2004) evaluated the role of TACE in acute inflammation using an inhibitor of the enzyme in a rat model of lung transplantation. Inhibition of this protease results decreased neutrophil accumulation in the alveolar space and other histological changes such as intercellular adhesion molecule-1 (ICAM-1) expression. In addition, significantly lower levels of monocyte chemotactic protein-1 (MCP-1), cytokine induced neutrophil chemoattractant-1 (CINC-1), high mobility group box-1 (HMGB1), and soluble epithelial cadherin and decreased neutrophil elastase activity were observed in bronchoalveolar lavage fluid from the rats treated with the inhibitor.

5 Mast Cell-Derived Proteases

Proteases are the most abundant class of proteins produced by mast cells. Many of these are stored in membrane-enclosed intracellular granules until liberated by degranulating stimuli, which include cross-linking of high affinity IgE receptor F(c) εRI by IgE bound to multivalent allergen [102]. It has been investigated that b-tryptase, a major protease released during mast cell activation, cleaves IgE and this tryptase-mediated IgE cleavage affects IgE binding to allergens [103]. From their study, IgE degradation products were detected in tryptase-containing tissue fluids collected from sites of allergic inflammation [103] and it has been confirmed that tryptase cleaves IgE and abolishes binding of IgE to allergens and F(c)εRI. It is a natural mechanism for controlling allergic reactions is supported by experiments performed with purified proteins, as well as by cellular *in vitro* and *in vivo* data [103].

The lung epithelial cells could be activated by airborne proteases from molds, mites, or pollens. Activation of other cells in the airway by various endogenous and exogenous proteases that increase production of IgE antibody and enhance infiltration of eosinophils, basophils, neutrophils, monocytes, and lymphocytes. Apart from that, smooth muscle contraction is enhanced, nerves are made more reactive, and airway responsiveness is increased [104]. Eosinophils and mast cells are degranulated and stimulated to produce inflammatory molecules, such as nitric oxide, major basic protein, leukotrienes, histamine, and mast cell tryptase itself. Mast cell tryptase is likely to be especially important in the late phase of the allergic response [105].

6 Proteases Involvement in Disease Progression

6.1 Chronic Obstructive Pulmonary Disease (COPD) and Pulmonary Emphysema

Chronic obstructive pulmonary disease (COPD) represents a group of diseases, including chronic bronchitis and emphysema, which are characterized by an airflow limitation that is not fully reversible [106]. The pathogenic roles of NSPs in COPD are attributed to their ability to break down connective tissue components and generate proinflammatory peptides from these components [107, 108], to induce mucus secretion by submucosal glandular cells and goblet cells, and to express proinflammatory cytokines from airway epithelial cells [109–111]. Pulmonary emphysema is a destructive lesion of the lung parenchyma. It has been demonstrated that the lesion of the matrix which results in emphysema is the end-result of crosstalk between macrophage metalloelastase and neutrophil elastase [112, 113]. However, the latter protease is responsible for the greater portion of the final proteolytic attack [112].

We have already mentioned before the role of MMP-12 in the pathogenesis of COPD. The most well studied MMPs in human COPD and emphysema are MMP-1, MMP-8, MMP-9, and MMP-12, which all have been implicated in tissue destruction in human COPD and emphysema [114]. The lymphocytes present in emphysematous lungs have a strong Th1 bias, expressing higher levels of CXCL10 (IP-10) and CXCL9 (MIG). This CXCL9 can upregulate the expression of MMP-12 in pulmonary macrophages [115], providing a mechanism for chronic and progressive destruction of lung parenchyma that is seen in emphysema.

6.2 Interstitial Lung Disease and Idiopathic Pulmonary Fibrosis (IPF)

Idiopathic pulmonary fibrosis (IPF), one of the most common forms of interstitial lung diseases, is a progressive fibrotic lung condition of unknown etiology [116]. The diagnosis of IPF is made by surgical lung biopsy, and the histopathological features include the presence of patchy inflammatory cells, foci of proliferating fibroblasts and myofibroblasts, and collagen deposition [117, 118]. From a study it has been observed that MMP-2 activity is increased in a dose-dependent manner in A549 cells treated for 48 h by TGF-β stimulation [119] because TGF-β plays a key role in stimulation of fibroblast proliferation and has been implicated in progression of IPF [120].

It has been postulated that NE may be involved in the early stages of lung inflammation during the development of pulmonary fibrosis [121]. Neutrophil elastase acts as a putative link between emphysema and fibrosis and this dual role of NE has been reported from a recent study that has highlighted NE as a common pathogenic mechanism linking pulmonary emphysema and fibrosis [122]. This study was done

in two animal models in which emphysema and fibrosis were induced either by bleomycin (BLM) or by chronic exposure to cigarette smoke. In order to study whether BLM-induced lesion is protease-dependent or not, a group of mice was treated with 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (a serine proteinase inhibitor active against neutrophil elastase). In DBA/2 mice that develop both emphysema and fibrosis after chronic cigarette-smoke exposure, the presence of NE in alveolar structures is also associated with a positive immunohistochemical reaction of both TGF-β and TGF-α [123]. These results strongly suggest that neutrophil elastase may be a common pathogenic link between emphysema and fibrosis, acting as a regulatory factor in the generation of soluble cytokines with mitogenic activity for mesenchymal cells resulting either in emphysema or in fibrosis or both [122]. A recent study has provided evidence that different interstitial levels of NE burden in emphysema may be associated with different routes of collagen clearance (intracellular vs. extracellular) and different degrees of remodeling of the ECM in emphysema [124]. This point merits further investigation. The implication of NE in lung destruction and repair and its pathogenic role in emphysema and fibrosis could lead to a novel approach for therapeutic interventions.

6.3 Cystic Fibrosis (CF)

Cystic fibrosis (CF) is a common, inheritable genetic disorder that results in malfunctioning of the chloride channels of many exocrine epithelial linings including the airway epithelia [125]. The exact mechanism or pathophysiology of progressive lung inflammation in patients with CF is not clear, but patients with CF have increased levels of MMP-2, MMP-8, and MMP-9 in their BAL [126]. In a study of 23 children with CF, BAL fluid concentrations of MMP8 and MMP9 were higher in untreated children and lower in those who were treated with DNase [127]. In children with stable CF, there is a significant inverse relationship between MMP-9 and lung function, as measured by FEV1. Furthermore, levels of MMP-9 are higher in sputum of asymptomatic children with CF compared with controls, suggesting that MMP-9 and total neutrophil count may be useful markers of airway injury and airflow obstruction in persons with CF [126]. As discussed in Sect. 7, MMPs play a critical role in the host defense against pathogens in the lung.

Neutrophils express three closely related serine proteases, NE, CG and PR3, in a coordinated fashion. It has been reported that IL-6 is susceptible to cleavage by all three proteases [128] but now a days, degradation of SIL-6R in a time and dose dependent manner has also been proved in the context of cystic fibrosis [129]. It has been studied that on the basis of molar concentration, among these proteases, CG is the most potent protease with maximal degradation occurring within 60 min at a concentration of 250 nM. NE also shows potent activity at 250 nM but required up to 4 h for complete degradation of sIL-6R to occur. PR3 was the least active protease with minimal degradation of sIL-6R occurring in the presence of 1,000 nM protease after 1 h and complete degradation being observed after 4 h in the presence of 500 nM of protease. If we consider CF lung, unregulated proteolytic activity is

the main contributor to pathology of this disease and is also responsible for reduced expression and activity of a number of important components of the immune response [130]. Targeting of IL-6 by NSPs [128] has previously been suggested as an explanation for the surprisingly low pulmonary expression of IL-6 in CF patients [131] however, this is the first study to show specific proteolysis of this cytokine by serine proteases in CF BALF [129].

6.4 Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS)

These are inflammatory disorders of the lung most commonly caused by trauma, sepsis, and pneumonia, the latter two being responsible for approximately 60 % of cases [132]. Early in the initiation of ALI and ARDS, massive number of neutrophils accumulates in the vasculature of the lung. Neutrophils and their cytotoxic products, including oxidants and proteases, have main pathological importance in ALI and ARDS. It has been observed that there is increased elastolytic activity [133] in patients with ARDS [134]. Increased levels of HNE in plasma and in BAL have also been observed with at-risk patients who later developed ALI [135, 136]. Pathologic effects of HNE are associated with microvascular injury, causing endothelial damage, increased capillary permeability, and interstitial edema. HNE may also potentiate the inflammatory response by increasing the expression and release of cytokines [137] and by increasing mucin production [138]. In experimental animal models, intratracheal administration of exogenous HNE induces lung hemorrhage and ALI, whereas administration of pharmacological HNE inhibitors prevents lung injury, which further supports the role of NSPs in lung injury [22, 139, 140]. Owen *et al.* (2004) propose an interesting role of MMP-8 that it may be anti-inflammatory during acute lung injury, because MMP-8 null mice given intratracheal LPS have significantly greater accumulation of neutrophils in the alveolar space than wild-type mice [141].

In patients with acute respiratory distress syndrome, salbutamol (b2-agonist) increases MMP-9 activity in bronchoalveolar lavage fluid, and increases MMP-9 but decreases TIMP-1 and -2 expressions in distal lung epithelial cells [142]. Similar findings are reported for formoterol (b2-adrenoceptor agonist) in a rat model of pulmonary inflammation [143]. From another study of Zhang *et al.*, an interesting data found that formoterol and ipratropium bromide partially protect the lungs against the inflammation by reducing neutrophilic infiltration. This protective effect is associated with reduced MMP-9 activity known to play an important pro-inflammatory role in acute inflammatory process [143].

6.5 Asthma

Asthma is characterized by episodic dyspnea, lung inflammation, and in some patients, progressive irreversible airway dysfunction [144]. Expression of several MMPs has been associated with asthma; enhancement of MMP-1, MMP-2, MMP-3,

MMP-8, and MMP-9 all have been found in sputum and BAL from patients with asthma [69]. With severe asthmatic conditions, patients have increased MMP9 activity in BAL relative to that from patients with mild asthma or control patients. Wenzel *et al.* [145] found an increase in MMP-9 activity in the subepithelial basement membrane that is accompanied by higher TGF-β. These studies suggest that in patients with severe asthma, neutrophils play a key role in lung remodeling because they express both MMP-9 and TGF-β, which are involved in breakdown and repair of tissue, respectively. In asthma, MMP-9 is expressed in bronchial epithelium and submucosa, where its abundance correlates with tissue eosinophil number [146] because MMP-9 is also produced by eosinophils, macrophages, and neutrophils [147, 148]. In sputum, concentration of MMP-9 is positively correlated with neutrophil number [149] and also with the cumulative macrophage, neutrophil, and eosinophil count [150] during asthma. BAL taken from asthmatic patients after allergen challenge increases the mitogenic indices even more than a normal patient's BAL [151]. This phenomenon could contribute to the airway remodeling observed in patients with chronic asthma; however, the pathway underlying the proliferation remains unclear. EGFR, TGF-β, or platelet-derived growth factors are all candidates based on their presence in lung during disease and their relationship to various MMPs [152].

Asthmatic patients express an increased amount of PAR-2 on respiratory epithelial cells but not on smooth muscle or alveolar macrophages [153, 154]. Schmidlin and colleagues [155] have studied the effect of PAR-2 on ovalbumin challenge of immunized mice. Compared with wild-type animals, eosinophil infiltration was inhibited by 73 % in mice lacking PAR-2 and increased by 88 % in mice over expressing PAR-2. Similarly, compared with wild-type animals, airway hyperreactivity to inhaled methacholine was diminished by 38 % in mice lacking PAR-2 and increased by 52 % in mice over expressing PAR-2. PAR-2 deletion also reduced IgE levels to ovalbumin sensitization 4-fold compared with levels seen in wild-type animals. Mast cell chymase induces eosinophil infiltration, presumably by activating PAR-1 [156]. Secretary leukocyte protease inhibitor administered intratracheally before allergen challenge prevented bronchoconstriction, airway hyperresponsiveness, and leukocyte influx [157]. The actions of PARs and their activating proteinases in the airways have also been studied extensively to determine their role in various lung diseases [158].

6.6 Pseudomonas aeruginosa Infection in Lung

Pseudomonas aeruginosa (Pa) is an opportunistic pathogen that infects over 80 % of CF adult lungs [159] and is a major cause of ventilator-associated pneumonia (VAP) in hospitalized patients [160]. A broad spectrum of Pa virulence factors has been identified including proteases secreted by various mechanisms [161]. Proteases such as elastase (LasB), alkaline protease (AprA), staphylolysin (LasA) and Protease IV (a serine-endoprotease) have been identified in CF lung [162, 163]. There are other virulence factors (proteases) of Pa which are capable to degrade host

proteins, including matrix components and components of the immune system such as immunoglobulins and serum alpha proteins [164]. The most studied Pa protease is the elastolytic metalloproteinase LasB, a type II secretion system enzyme. There are several functions of Pa elastase has been reported such as it can alters epithelial barrier function [165], disables PAR-2 receptor in lung epithelial cells [166], cleaves uPAR leading to disruptions of uPAR-dependent cellular interaction [167], inactivates complement and immunoglobulins, and inhibits cell chemotaxis, phagocytosis and microbicidal activities in human leukocytes [168, 169].

The present findings reveal a protective role for extracellular NE against the pathogen. It has been demonstrated that NE, an endogenous effecter, could also participate in the orchestration of lung inflammatory response against *P. aeruginosa* infection by modulating the expression of cytokines (e.g., induction of the expression of the pro-inflammatory TNF-α, MIP-2, and IL-6) [5].

6.7 Allergic bronchopulmonary Aspergillosis

Allergic Bronchopulmonary Aspergillosis (ABPA) occurs in nonimmunocompromised patients and belongs to the hypersensitivity disorders induced by *Aspergillus* sp. Genetic factors and activation of bronchial epithelial cells in asthma or cystic fibrosis are responsible for the development of a CD4 + Th2 lymphocyte activation and IgE, IgG and IgA-AF antibodies production [170]. It appears that total serum IgE levels are extremely high, and not all of it is specific antibody to *Aspergillus* species antigens. The pathological changes such as pulmonary infiltrates of eosinophilic pneumonitis, granulomatous central bronchiectasis, and segmental pulmonary fibrosis are seen [171]. *Aspergillus* species produces proteases [172–176] which can desquamate epithelial cells and stimulate IL-6 and IL-8 production [172]. *Aspergillus* species proteases also initiate growth factor release from epithelial cells and are possibly responsible for the central bronchiectasis [177]. It still remains unclear what is abnormal about the response in the few patients in whom this disease develops and why it is especially common in individuals with cystic fibrosis.

7 Conclusions

As we have mentioned before that generally three major classes of proteases—serine proteases, MMPs, and cysteinyl proteases—have been identified in the lung. They are associated traditionally with various inflammatory lung diseases and airway extracellular matrix destruction. From ample evidences it has been proved that each protease family has a multitude of regulatory functions, which makes them of pivotal importance in inflammation, innate immunity, and infection. For example, if we consider role of NE in lung inflammation, recent observations suggest that it's role is more complex than the simple degradation of ECM components. Several

lines of evidence propose that NE aims specifically at a variety of regulatory functions in local inflammatory processes but their relevance under various pathophysiological conditions still remains poorly understood and need further investigation. NE, acting as a link between pulmonary emphysema and fibrosis that could lead to a novel strategy for therapeutic interventions.

Among various lung inflammatory diseases, in case of chronic infective lung diseases, the normal physiological processes become deregulated because of extracellular protease activity, which leads ultimately to upregulation of proinflammatory mediators, increased recruitment of inflammatory cells to the lung, impaired phagocytosis, enhanced mucin production, and inactivation of important innate and antimicrobial proteins. This results in sustained inflammation and predisposition to infection. One way to treat such protease-mediated events in chronic infective lung disease is with antiprotease therapy, which neutralizes excessive extracellular protease activity without compromising the normal physiologic role of proteases. Antiprotease trials have been performed using α_1-antitrypsin and secretary leukoprotease inhibitor, both of which have successfully inhibited NE activity *in vivo* in CF and α1-antitrypsin deficiency [178–180]. Other inhibitors of serine proteases and MMPs are being developed, including synthetic inhibitors to combat protease-induced lung destruction [181].

As these NSPs participate in a variety of pathophysiological processes, they appear as potential therapeutic targets for drugs that inhibit their active site or impair activation from their precursor. Overall, the available preclinical and clinical data suggest that inhibition of NSPs using therapeutic inhibitors would suppress or attenuate deleterious effects of inflammatory diseases, including lung diseases [182].

MMPs compromise a structurally and functionally related group of proteolytic enzymes, which play a key role in the tissue remodeling and repair associated with inflammation [183]. Among various MMPs, MMP-12 has massive importance for lung remodeling in patients suffering from COPD, and its importance was confirmed in MMP knockout mice that were protected against smoke-induced emphysema [184]. Presently it has been found that MMP-12 activity was higher in ex-smokers with COPD compared with that seen in smokers with COPD, and this suggests that the smoking effect keep increasing MMP-12 activity, is irreversible and more severe disease can be associated with deregulated MMP-12 function [74]. Other MMP such as MMP-2 would be the target for treatment of cadmium induced lung inflammation [76]. Animal models of human lung diseases and clinical studies have provided ample evidences for the involvement of MMPs in the development or progression of a number of common lung diseases such as COPD, emphysema, and asthma. However, merely detecting increases in MMPs expression in various lung inflammatory conditions may not provide sufficient information to understand the contribution of MMPs in human lung diseases [152].

Metalloproteinases of the ADAM family have been recognized as potential therapeutic targets in several diseases. However, due to their broad activity, systemic application of inhibitors may not represent an appropriate treatment strategy. ADAM17 mediates a number of shedding events that influence several components of acute lung inflammation including vascular leakage, leukocyte recruitment and

cytokine release. Particularly in endothelial cells, ADAM17 appears to act as a central regulator in pulmonary inflammation. So, in near future inhibition of ADAM17, possibly locally to reduce systemic side effects, may be a promising approach for the treatment of Acute Lung Injury [100].

Given that they are involved in the pathogenesis of various diseases, they can be good therapeutic targets along with their specific protease inhibitors [185]. There could be a further option of combining protease modulators with monoclonal antibodies against cytokines to treat such inflammatory lung diseases. From clinical point of view whether anti-protease treatment or use of these proteases along with their specific inhibitors for curing these inflammatory lung diseases is beneficial, needs further investigation and research.

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Involvement of Proteolytic Enzymes in Cardiac Dysfunction Due to Ischemia-Reperfusion Injury

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Abstract It is well known that cardiac dysfunction in ischemic heart disease is associated with myocardial cell damage, apoptosis and activation of proteolytic enzymes including matrix metalloproteases (MMPs), cathepsin, calpains and caspases. These alterations due to ischemia-reperfusion (I/R) injury are mainly elicited by the occurrence of oxidative stress and the development of intracellular Ca^{2+}-overload in the heart. Depression in the activities of endogenous inhibitors such as tissue inhibitors of metalloproteases increases the activity of MMPs whereas that of calpastatin augments the activity of calpains. Endothelial dysfunction associated with depressed formation of nitric oxide (NO) due to I/R injury has also been shown to increase the activity of calpain. While the activation of MMPs and cathepsin is mainly focused with the degradation of extracellular matrix proteins, the activation of calpain and MMP-2 has been reported to degrade sarcolemmal, sarcoplasmic reticular, mitochondrial and myofibrillar proteins. The activation of caspases has also been demonstrated to induce apoptosis in the ischemic-reperfused heart. These defects in the structure and function of subcellular organelles induced by protease activation are suggested to result in contractile abnormalities in the heart due to I/R injury.

Keywords Ischemia-reperfusion • Oxidative stress • Intracellular Ca^{2+}-overload • Subcellular defects • Activation of proteases • Matrix metalloproteases • Calpain activation

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1 Introduction

The pathophysiology of cardiac dysfunction due to ischemic heart disease involves atherosclerosis and thrombosis of the coronary artery, which impair and block blood flow to the myocardium. Furthermore, the restoration of coronary flow to the ischemic heart, if not instituted within a certain period of ischemic insult, is known to be associated with a depression in cardiac contractile function and is generally referred to as ischemic/reperfusion (I/R) injury [1–4]. In fact, reperfusion of the ischemic heart has been shown to produce changes in subcellular organelles such as the sarcolemma (SL) [5–8], sarcoplasmic reticulum (SR) [9, 10], myofibrils [5, 11] and mitochondria [5, 12]. A wide variety of mechanisms [13] including the occurrence of oxidative stress [14, 15] and the development of intracellular Ca^{2+}-overload [4, 6], which involve the activation of proteases [9, 16, 17], have been suggested to explain subcellular alterations and cardiac dysfunction as a consequence of I/R injury. Several studies have indicated that the genesis of intracellular Ca^{2+}-overload due to I/R injury is attributed to abnormalities in Ca^{2+}-transport channels in the SL, SR and mitochondrial membranes [18, 19]. Furthermore, it has been suggested that intracellular Ca^{2+}-overload results in the activation of various proteases including calpain [9, 16]. Thus, the proteolysis of subcellular proteins is considered to contribute to the SL and SR dysfunction [5, 9, 15]. In addition, the activation of calpain has been shown to induce abnormalities in mitochondrial function [12, 20] and contractile proteins [11, 21].

Although calpain is known to play an important role in normal physiologic processes at the basal Ca^{2+}-levels [22], an increase in the intracellular concentration of free Ca^{2+} has been reported to be associated with pathologic events as a consequence of the activation of calpain [18]. In fact, both SR Ca^{2+}-handling proteins [9, 17] and NO regulating synthase [23] have been shown to be the target of the calpain activation within the heart. Previous studies have revealed that SR and SL Ca^{2+}-cycling and regulating proteins are the targets for proteolytic action of Ca^{2+}-activated proteases such as calpains [9, 24–26]. It may be noted that a transient increase in intracellular Ca^{2+} has been reported to cause the activation of calpains [27]. While cardiac dysfunction induced by I/R injury may be a consequence of the proteolytic action of calpain due to the development of intracellular Ca^{2+}-overload, different investigators [14, 28–31] have suggested that the activation of matrix metalloproteases (MMPs) by oxidative stress may be important in inducing cardiac dysfunction in ischemic heart disease. The activation of MMPs has been shown to degrade the extracellular matrix proteins [28, 30]; however, MMP-2 and MMP-9 have also been implicated in degrading intracellular proteins [29, 31]. Extensive information concerning the involvement of different proteases in the genesis of cardiac remodeling and subcellular defects leading to heart failure has been reviewed recently [32, 33]. The present article is focused on reviewing the role of calpains and MMPs in inducing cardiac cell damage due to ischemic heart disease. Some efforts are made in discussing the mechanisms of protease activation due to I/R-injury. Furthermore, the role of calpain and MMP-2 in inducing subcellular defects and cardiac dysfunction due to I/R injury is described.

2 Activation of Different Proteases in Ischemic Heart Disease

Different types of enzymes (calpains, MMPs, cathepsins, ubiquitin-proteosomes) with proteolytic activity have been identified within the myocardium [30, 34–36]. Calpains are calcium-dependent cysteine proteases, which are involved in a variety of Ca^{2+}-regulated cellular processes including signal transduction, cytoskeletal remodeling, cell proliferation and differentiation, apoptosis, membrane fusion, platelet activation, cell cycle regulation, insulin secretion and sex determination [37, 38]. Several types of calpain exist but two ubiquitous isoforms that differ in their Ca^{2+} sensitivity are common; these are μ-calpain and m-calpain (named due to their micromolor and millimolar Ca^{2+} requirements). Calpain exists in the cytosol as an inactive enzyme and translocates to the cell membrane in response to an increase in the cellular Ca^{2+} level that occur transiently during ischemia and I/R, where it is further activated by the presence of Ca^{2+} and phospholipids. Calpain activation occurs by autolysis and is triggered by the association of Ca^{2+} to at least three different sites on the enzyme. Under normal physiological conditions, calpain activity is tightly regulated by Ca^{2+} because disorder in the calpain activity causes excessive degradation or accumulation of coexisting cellular proteins resulting in serious cellular damage under pathological conditions [37–41]. It is pointed out that calpastatin is a highly selective endogenous inhibitor of calpain, which is a heat-stable protein and is resistant to many denaturing agents such as urea and trichloroacetic acid [42, 43]. Calpastatin also inhibits other proteases like cathepsin B, trypsin, plasmin, thrombin, pepsin and various forms of cysteine proteases and is thus not specific in nature.

Earlier studies have demonstrated that calpain causes proteolytic modification of the SR Ca^{2+}-handling proteins including Ca^{2+}-pump ATPase, Ca^{2+}-release channel and phospholamban [9] as well as the SL proteins including Na^{+}-Ca^{2+} exchanger, Na^{+}-H^{+} exchanger and Na^{+}-K^{+}-ATPase during I/R injury [24, 44–47]. Calpain has been shown to degrade the membrane cytoskeleton proteins like fodrin (calspectin or non-erythroid spectrin) and ankyrins that maintain cell membrane integrity [17, 25]. It has also been found that degradation of α-fodrin and calpastatin occurs during ischemia and I/R injury [17]. Besides fodrin, other cytoskeletal proteins such as desmin and α-actinin are targeted by calpain. The reduction in degradation of these proteins seen with leupeptin, a calpain inhibitor, has been shown to correlate with improved cardiac contractility; this observation suggests an association between calpain degradation of the cytoskeletal proteins and cardiac contractility [48, 49]. It has been claimed that fodrin is probably the only cytoskeletal protein to be degraded in I/R-induced proteolysis, as there was no evidence for the proteolysis of other proteins under the experimental conditions of this study [50]. On the other hand, some investigations have shown that Ca^{2+}-release channel of the SR [51] and Ca^{2+}-pump ATPase [26] and L-type Ca^{2+} channel [52] are the possible targets of calpain. Intracellular Ca^{2+} overload in patients with atrial fibrillation was also found to be associated with calpain mediated proteolytic degradation of atrial proteins and this alteration in the structural integrity of the sarcomere was implicated in the pathogenesis of serious arrhythmias [53].

Various studies have reported that Ca^{2+} sensitivity of myofilaments is reduced in I/R injury [54, 55]. Ca^{2+} responsiveness of skinned muscle fibres was decreased when exposed to calpain I [56], suggesting that calpain activation during I/R may be responsible for altering the myofilament structure and response to Ca^{2+}. Calpain induced degradation of contractile protein troponin T (TnT) [11] results in the persistence of immunoreactivity of TnT in the serum of patients recovering from acute myocardial infarction (MI). In fact, measurement of the plasma level of cardiac TnT has emerged as one of the current diagnostic tools for establishing the early diagnosis of MI [57, 58]. Cross-linked proteins have also been suggested to be responsible for the persistent immunoreactivity observed in MI patients. Uncontrolled Ca^{2+}-dependent calpain activation has been implicated in several other pathological conditions including cerebrovascular disease and Alzheimer's disease [31, 59–61]. In addition, calpains have been reported to be involved in a wide range of pathological states including those associated with genetic mutations such as type II diabetes (which results from a mutation in calpain 10, CAPN10), Duchenne's muscular dystrophy and Parkinson's disease [62, 63].

Another family of proteolytic enzymes found in the myocardium and implicated in ischemic heart disease is MMPs. These proteases exist in an inactive zymogen state under normal conditions and are activated by proteolysis, nitrosylation, glutathiolation, phosphorylation and oxidative stress [30, 64, 65]. A significant amount of MMP-2 has been shown to be present in cardiomyocytes; this is activated by oxidative stress mediated through peroxynitrite [30]. MMP-2 is located intracellularly in cardiac myocytes and is co-localized with troponin I within myofilaments as well as in the nucleus in an inactive state. The activation of MMP also occurs through limited proteolysis by trypsin and elastase [66] and is dependent upon two key promoters namely, furin and membrane type 1-MMP (MT-MMP-1). Furin (a subtilisin/kexin-like pro-protein convertase), which is a serine endopeptidase located in the trans golgi apparatus region of cells including cardiomyocytes. It is crucial for the activation of MMPs especially MT-MMP-2 and is responsible for fibroblast migration. Furin activates MMPs subsequent to ischemia and causes extracellular matrix degradation and thus increases the fibroblast migration. Similarly, MT-MMP-1 and MMP-14 are proteases, which are activated by furin and located in the golgi apparatus; upon activation, these proteases translocate to the membrane [67]. MT-MMP-1 activates MMPs causing increased extracellular matrix and vascular endothelial basement membrane degradation. Tissue growth factor β1 has also been shown to increase the MT-MMP-2 activity in cardiac fibroblasts thereby increasing their migration [68, 69]. Tissue inhibitor of metalloproteases (TIMP) is an endogenous inhibitor of MMPs and reduction in its activity has been shown to activate MMPs in the myocardium due to I/R injury [16, 29, 31, 32].

In addition to MMPs, different proteases such as calpains and caspases are involved in necrotic and apoptotic cell death as a consequence of the ischemic insult [31]. It should be mentioned that necrosis is a rapid, irreversible process that occurs when cells are severely damaged and involves ATP depletion, loss of cation gradients and membrane rupture [70, 71]. Apoptosis, on the other hand, is a programmed, highly organized, energy dependent mechanism whereby a cell commits suicide upon DNA fragmentation without causing damage to the surrounding tissue [72].

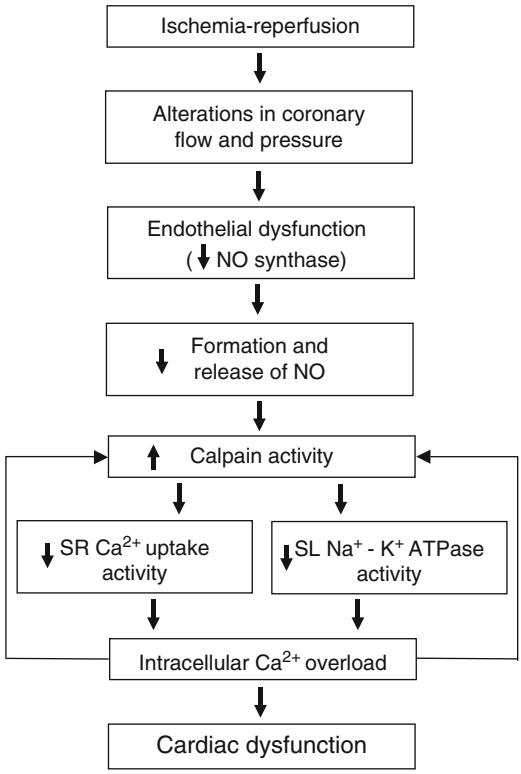

Fig. 1 Proposed sequence of events involving endothelial defect, calpain activation and depressed subcellular activities during the development of ischemia-reperfusion induced cardiac dysfunction. SR, sarcoplasmic reticulum; SL, sarcolemma

Apoptosis occurs in cardiac tissue during ischemia and/or reperfusion and is mediated by a family of cysteine-dependent aspartate specific proteases called caspases [73]. This is believed to occur due to the activation of plasma membrane death receptors and/or translocation of Bcl-2 homologous proteins to mitochondria. This change is associated with increased mitochondrial permeability transition, cytochrome c release, caspase activation and contractile dysfunction. Although cardiomyocytes undergo caspase-independent 'autophagic' death [74, 75], apoptosis plays a pivotal role in the development of septal, valvular and other vascular structures as well as various congenital cardiac diseases [76]. Since apoptosis has been shown to induce endothelial NO synthase (eNOS) deficiency causing heart failure and congenital septal defects [77], it is likely that endothelial dysfunction and activation of some proteolytic enzymes may be involved in this event. It should be pointed out that endothelial function with respect to NO production has been observed to be defective in ischemic heart disease [78, 79]. In fact, some studies have revealed a close association of endothelial dysfunction, reduction in NO synthase content, depression in NO formation, increase in calpain activity, subcellular defects and impairment in cardiac performance due to I/R injury [23, 80]. A schematic representation of the involvement of endothelial dysfunction in the I/R-induced activation of calpain and subsequent subcellular changes leading to cardiac dysfunction is shown in Fig. 1.

3 I/R-Induced Changes in Subcellular Activities and the Activation of Proteases in the Heart

It has been reported that the I/R-induced depression in cardiac function is associated with a decrease in the SL Na^+-K^+ ATPase activity, whereas oxidative stress and intracellular Ca^{2+}-overload are considered to contribute to alterations in the Na^+-K^+ ATPase isoforms [7, 8, 61]. We have also demonstrated that Na^+-K^+ ATPase isoforms are targets for the action of calpain during the development of I/R injury [24, 61]. It is believed that an increase in the intracellular concentration of Ca^{2+} during I/R is sufficient to cause the activation of calpain isoforms 1 and 2 in the heart [81]. Calpain activation due to its translocation has been observed in cancer cells upon activation by Ca^{2+} [82] as well as in cardiac cells upon subjecting to I/R injury [24]. The development of intracellular Ca^{2+}-overload in the I/R heart has been reported to activate calpain, degrade different subcellular proteins, depress the activities of various subcellular organelles and result in the impairment of cardiac function [9, 23, 24, 61]. On the other hand, the occurrence of oxidative stress in the I/R heart has been shown to increase the activity of MMPs, degrade different proteins in the extracellular matrix and produce cardiac dysfunction [28, 29, 31, 83]. Various studies have also identified the localization of MMP-2 in cardiomyocytes and the activity of this proteolytic enzyme has been shown to increase in hearts subjected to I/R as well as oxidative stress [29, 30, 84–86]. In fact, different subcellular proteins have been reported to be the targets of MMP-2 and thus the activation of intracellular MMP has also been suggested to explain subcellular defects and cardiac dysfunction due to I/R [83, 87, 88]. Previous studies from our laboratory have demonstrated that both calpain and MMP-2 are activated in hearts subjected to I/R and both oxidative stress and intracellular Ca^{2+}-overload play a critical role in the activation of these proteolytic enzymes as well as subcellular defects [9, 23, 24, 33].

The relationship among the activation of proteases, subcellular defects and cardiac dysfunction was examined in isolated perfused hearts subjected to I/R injury. I/R-induced activation of both calpain and MMP-2 activities and depression in cardiac contractile function were associated with decrease in myofibrillar Ca^{2+}-stimulated ATPase, SR Ca^{2+}-pump and SL Na^+-K^+ ATPase activities [9, 21, 24, 61]. The I/R-induced changes in cardiac function, Na^+-K^+ ATPase and calpain activities were dependent upon the extracellular concentration of Ca^{2+} in the perfusion medium [24]. Perfusion of the hearts with leupeptin, an inhibitor of calpain, was observed to attenuate changes in myofibrillar Ca^{2+}-stimulated ATPase, SR Ca^{2+}-pump and SL Na^+-K^+ ATPase activities [9, 21, 24]. The I/R-induced alterations in cardiac function and calpain as well as SL Na^+-K^+ ATPase activities were also attenuated [24] by MDL 28170, a specific inhibitor of calpain [46, 89]. It should be noted that the I/R-induced depressions in SL Na^+-K^+ ATPase activity as well as SL Na^+-K^+ ATPase α_1, α_2, β_1 and β_2 subunits were not only attenuated by leupeptin, but were also simulated upon incubation of the SL membrane with activated calpain [24]. Furthermore, the I/R-induced increase in calpain activity due to I/R was associated with translocation of both calpain 1 and calpain 2 isoforms as well as changes in the

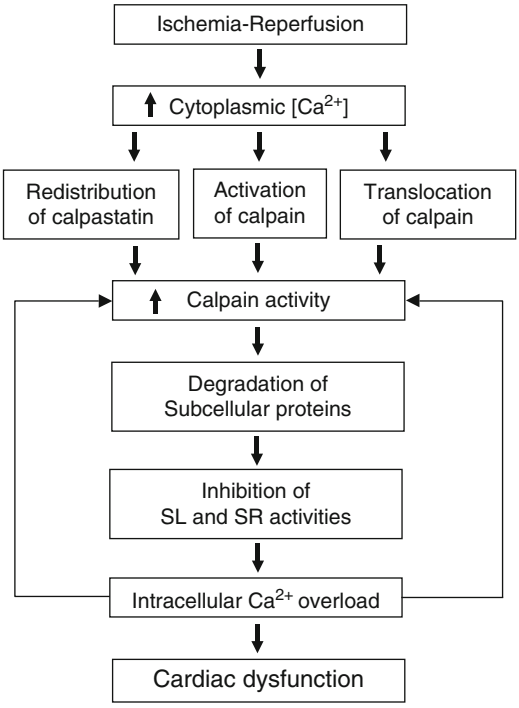

Fig. 2 Proposed sequence of events involving intracellular Ca^{2+}-overload, activation of calpain and inhibition of subcellular activities leading to ischemia-reperfusion induced cardiac dysfunction. SL, sarcolemma; SR, sarcoplasmic reticulum

distribution of calpastatin, an endogenous inhibitor of calpain [90]. These alterations in cardiomyocytes were prevented by perfusing the hearts with MDL 28170 [24]. In view of the role of intracellular Ca^{2+}-overload in the pathogenesis of cardiac dysfunction due to I/R [5, 6], it is evident that the activation of calpain due to elevated levels of intracellular Ca^{2+} may inhibit subcellular activities by degrading the subcellular proteins. The involvement of calpain activation due to intracellular Ca^{2+}-overload in inducing subcellular defects and cardiac dysfunction due to I/R is depicted in Fig. 2. Additionally, the I/R-induced increase in calpain activity is not associated with any change in protein content of calpain 1 and calpain 2 isoforms, whereas that of calpastatin were decreased [21].

Not only does the activation of calpain play a critical role in I/R-induced cardiac injury [9, 24, 91], activation of MMPs is also involved in inducing defects in extracellular matrix and subcellular organelles in the I/R hearts [28, 29, 31, 87, 92]. The involvement of calpain activation [45, 93, 94] and MMP-2 activation [29, 95–97] in I/R-induced functional and structural abnormalities in the heart have also been reported. Recent studies from our laboratory have suggested that both calpain and MMP-2 are activated upon subjecting the heart to I/R injury and these changes are associated with depression in Na^+-K^+ ATPase activity as well as protein content for α_1, α_2, β_1 and β_3 subunits [24, 60, 61]. Perfusion of the heart with doxycycline, an inhibitor of MMP-2 activity [87], was found to attenuate I/R-induced cardiac dysfunction, degradation of myosin light chain as well as changes in the activity of

Na^+-K^+ ATPase and protein content of its subunits [60, 61]. The increased activity of MMP-2 in the I/R heart has been observed to be associated with a depression in protein content for the endogenous inhibitor of MMPs [88]. It is pointed out that perfusion of the heart with doxycycline attenuated the I/R-induced activation of MMP-2 without affecting the elevated levels of calpain, whereas both leupeptin and MDL 28170 produced a marked depression in the activation of calpain and a significant reduction in the MMP-2 activity in the I/R hearts [61]. Accordingly, it was suggested that activation of MMP-2 may partly be due to the increased calpain activity in the I/R hearts. Other proteolytic enzymes such as cathepsins have also been observed to induce the activation of extracellular MMPs [98, 99]. Global ischemia has also been reported to increase the activities of both calpain and MMP-2 in addition to inducing subcellular alterations and changes in Na^+-K^+ ATPase subunits [60].

Subjecting the heart to different times of global ischemia as well as I/R revealed that the magnitude of calpain activation was greater than that of MMP-2 activation [60]. Furthermore, inhibition of calpain by MDL 28170 was more effective in attenuating the I/R-induced alterations in cardiac contracture, Na^+-K^+ ATPase activity and α_2 subunit protein content than the inhibition of MMP-2 by doxycycline [60]. While the I/R-induced activation of calpain has been shown to be primarily due to the occurrence of intracellular Ca^{2+}-overload [24, 31], the activation of MMP-2 has been reported mainly due to oxidative stress [29, 97, 100]. We have observed that I/R-induced changes in both calpain and MMP-2 activities as well as cardiac function and subcellular activities were simulated by reperfusion of the hypoxic hearts with conditions which are known to promote oxidative stress [61]. Likewise, perfusing the hearts with the oxyradical generating system, xanthine plus xanthine oxidase, as well as with a well known oxidant, H_2O_2, also produced alterations similar to those seen in I/R hearts [61]. Ischemic preconditioning, which is known to reduce oxidative stress, was found to attenuate I/R-induced activation of proteolytic enzymes as well as changes in cardiac function and subcellular activities [5, 15, 61]. In addition, different anti-oxidants including N-acetylcysteine and mercaptopropionylglycine, were also observed to prevent I/R-induced changes in Na^+-K^+ ATPase, calpain and MMP-2 activities as well as cardiac function [61]. These observations support the view that oxidative stress is intimately involved in the activation of calpain and MMP-2 enzymes as well as other subcellular changes due to I/R. A schematic diagram illustrating the involvement of oxidative stress, activation of proteolytic enzymes and subcellular defects in I/R-induced cardiac dysfunction is shown in Fig. 3.

4 Conclusions

From the foregoing discussion, it is evident that proteolytic enzymes including calpains and MMPs are activated in the heart due to I/R. Several mechanisms including reduction in NO_2 production, imbalance of endogenous inhibitors, occurrence of intracellular Ca^{2+}-overload and the development of oxidative stress seem to account

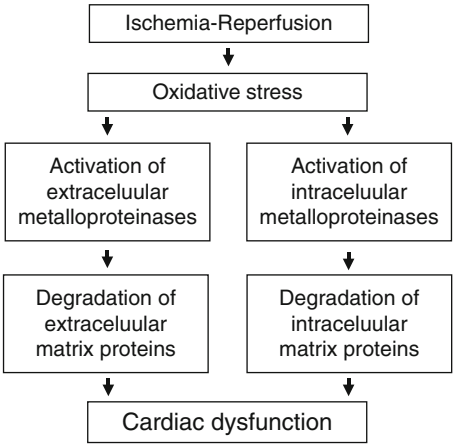

Fig. 3 Proposed sequence of events involving oxidative stress, activation of both extracellular and intracellular metalloproteinases (proteases) and degradation of extracellular matrix proteins and intracellular (subcellular) proteins leading to cardiac dysfunction

for the activation of these proteolytic enzymes upon subjecting the heart to ischemic and I/R insults. The degradations of extracellular matrix and subcellular proteins including collagen, myofibril, SR, SL and mitochondrial proteins due to I/R-induced activation of proteolytic enzymes appears to result in cardiac dysfunction. The I/R-induced apoptosis associated with cardiac dysfunction has been demonstrated to be due to mitochondrial defects and activation of caspases. It is therefore proposed that inhibitors of proteolysis may serve as the most suited therapeutic agents for the treatment of ischemic heart disease.

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