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Protease Inhibitors: Role and Potential Therapeutic Use in Human Cancer

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Proteases and protease inhibitors have been increasingly recognised as important factors in the physiopathology of human diseases, and our understanding of their role in cancer has dramatically increased over the last decade. We have obtained causal evidence linking proteases to tumour invasion and metastasis, and have become aware of genuine mechanisms used by tumour cells to optimise the use of proteases in the pericellular matrix. Many synthetic and natural inhibitors of these proteases have also been characterised, and their mechanisms of interaction with their corresponding enzymes are progressively unveiled as the X-ray crystal structures of these enzymes and their inhibitors are now reported. It has also become evident that many of these inhibitors, in addition to preventing the dissemination of cancer cells, have an inhibitory effect on tumour growth. Thus protease inhibitors are emerging as potentially therapeutic tools to treat cancer. In this article, recent studies on the role of proteases and their inhibitors in cancer are reviewed, and current ideas on their potential use as therapeutic agents are discussed.

Key words: proteases, protease inhibitors, invasion, metastasis, angiogenesis
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PROTEASES AND THEIR INHIBITORS IN HUMAN DISEASES

PROTEASES PLAY a key role in many physiological processes such as blood coagulation and fibrinolysis, complement and cytokine activation, cell migration, organogenesis, trophoblastic implantation, or tissue remodelling, and have been increasingly identified as important factors in the pathophysiology of a large number of human diseases. Several pathological conditions including thrombotic disorders, hypertension, osteoarthritis, chronic degenerative diseases and cancer are caused by changes in protease activity [1–4], and many human pathogens rely on proteases to infect the host [5–7].

Table 1. Proteases in human diseases

Disorder	Examples	Protease	Protease inhibitor
Blood dyscrasia	Thrombosis	Thrombin	Hirudin, tPA, streptokinase
Chronic degenerative	Emphysema	Leucocyte elastase	α_1-antitrypsin
	Cystic fibrosis		
Neurological	Alzheimer's disease	Metalloproteinases	n.d.
Inflammatory	Rheumatoid arthritis	Collagenase	TIMPs, hydroxamate
	Osteoarthritis	$IL_1\beta$ converting enzyme (ICE)	(ICE)
Infectious	Periodontal disease	Collagenase	Tetracyclines
	AIDS	HIV protease	Synthetic peptides
		Reverse transcriptase	Nucleoside analogues
	Bacterial infections	Penicillinase	Clavulanic acid
	Parasitic infections	Cysteine proteinase, haemoglobinase	Hydroxamate
Cardiovascular	Hypertension	Angiotensin converting enzyme (ACE)	Captopryl
Cancer	Invasion, metastasis tumour growth	Metalloproteinases	Batimastat (BB-94)

n.d., none described; TIMP, tissue inhibitor of metalloproteinases.

The activity of most extracellular proteases is controlled by specific natural inhibitors, and the importance of some of these inhibitors in human pathology is well illustrated by the identification of particular conditions associated with their deficiency. For example, deficiency in the thrombin inhibitor antithrombin III results in recurrent thrombotic disorders [8], while deficiencies in α_2-antiplasmin and plasminogen activator inhibitor-1 (PAI-1) are associated with haemorrhagic disorders [9] and deficiency in α_1-antitrypsin causes lung emphysema and chronic bronchitis [10, 11]. As our understanding of the role of proteases and protease inhibitors in human pathophysiology increases, inhibitors of proteases are emerging as novel and potentially superior agents for the prevention and treatment of many human conditions (Table 1). For example, Captopril, an inhibitor of the angiotensin-converting enzyme, is part of today's standard treatment of hypertension [12]. Other inhibitors such as α_1-antitrypsin [11, 13], Hirudin, a thrombin inhibitor [14], and Batimastat (BB-94), an inhibitor of metalloproteinases [15], are currently being tested in clinical trials, and the number of inhibitors tested in animal models is steadily growing. This article reviews recent studies on the role of proteases and their inhibitors in cancer, and presents current ideas concerning their use as potential anticancer agents.

OPTIMAL UTILIZATION OF PROTEASES BY CANCER CELLS

The role of proteases in cancer has been the subject of extensive investigations that are beyond the scope of this article and several comprehensive review articles have been published [16, 17]. Early studies suggested that extracellular proteases are essential for tumour cells to penetrate the basement membrane, a process that typically distinguishes a carcinoma *in situ* from an invasive carcinoma. Proteolytic degradation of the extracellular matrix (ECM) is also required when invasive tumour cells penetrate tissues, gain access to the blood circulation (intravasation), and exit blood vessels (extravasation) to colonise distant metastatic sites. Furthermore, angiogenesis—a neovascularisation process essential to sustain tumour growth—involves active proteolytic degradation of the ECM by invasive endothelial cells [18]. All members of the four major classes of endopeptidases including serine, cysteine, aspartyl and metalloproteinases have been implicated in these processes [16]. Although these families of proteases differ significantly in their structure, substrate specificity and active site, a common theme is their production in inactive pro-forms (zymogen) that require activation. Serine proteinases are a class of endopeptidases characterised by having a serine residue in the active site. This family includes many important peptidases such as trypsin, chymotrypsin, thrombin, plasmin, human leucocyte elastase, cathepsin G and urokinase type (uPA) and tissue type (tPA) plasminogen activators. uPA and tPA are produced by many tumour cells [19], and uPA can be concentrated at the surface of cells by the presence of a membrane-associated uPA receptor [20, 21]. Plasmin is generated by a single proteolytic cleavage at ^{860}Arg by uPA and tPA, and has a proteolytic activity directed toward several components of the ECM such as fibronectin, laminin and type IV collagen [22]. Plasmin is also an activator of several metalloproteinases, in particular interstitial procollagenase. As is the case for uPA, cells can concentrate plasminogen at their surface via a specific plasminogen/plasmin receptor [23]. Among cysteine proteinases, two proteases, cathepsin B and L, have been particularly implicated in cancer [24]. These lysosomal proteases, having a cysteine residue at their active site, have an optimal activity at acid pH but can degrade extracellular matrix proteins at neutral pH. They have a broad spectrum of substrates and are actively involved in the degradation of proteolytic products generated by other proteases [25]. Cathepsin B shares certain properties with uPA and can activate latent collagenase and receptor-bound uPA [24]. Cathepsin D is an aspartic protease that, like all members of the cathepsin family, is active at acid pH, and is primarily present in lysosomes where it degrades a large variety of endocytosed proteins. This

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protease has been particularly implicated in human breast cancer where high levels of expression were found to correlate with relapse and metastatic disease [26, 27]. Matrix metalloproteinases (MMPs) consist of a large family of at least 10 members [28, 29] including interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), two type IV collagenases (gelatinase A and B or MMP-2 and MMP-9), three stromelysins, matrilysin, a macrophage-derived metalloelastase, and a recently described membrane-type metalloproteinase (MT-MMP) [30]. These enzymes have the following features in common: (1) they are produced in an inactive pro-form; (2) they have two Zn^{++} atoms including one at the active site; (3) they have two Ca^{++} ions essential for the stability of the enzyme [31, 32]; (4) their primary structure typically contains two highly conserved regions, a PRCGV/NPD motif in the N-terminal propeptide domain, and a HEXGH motif in the catalytic domain; and (5) they are inhibited by a specific family of inhibitors designated tissue inhibitors of metalloproteinases (TIMPs). The spectrum of proteolytic activity of MMPs is broad and includes interstitial and basement membrane collagens, glycoproteins, proteoglycans and denatured collagen (gelatin). Some of these proteases, such as stromelysin and MT-MMP, can activate other pro-MMPs [30, 33]. In contast to uPA, no specific cellular receptor for MMP has yet been isolated and characterised. However, association of MMPs to plasma membrane has been shown [34], and the existence of a receptor binding the C-terminal domain of gelatinase A has been postulated [35, 36]. Furthermore, a novel MMP with a unique transmembrane domain has been recently described [30]. It is, therefore, likely that, like serine proteases, some MMPs can be specifically located at the cell surface via a specific membrane receptor.

Evidence supporting the role of proteases in cancer has been initially derived from experiments demonstrating a positive correlation between the proteolytic activity of mammalian tumour cell lines and their ability to either invade reconstituted basement membranes or artificial tissue substrates *in vitro* and/or to metastasise after injection in syngeneic or immunodeficient animals [37]. As specific antibodies and cDNA probes for these proteases became available, it has been possible to examine the production of these proteases in human tumour tissues using sensitive techniques, such as immunohistochemistry and *in situ* hybridisation, and to obtain more direct evidence of their implication in human cancer. In general, these studies have indicated the presence of a positive correlation between the expression of these proteases and the incidence of local recurrence, the presence of lymph node or distant organ metastasis and patient survival [38–44]. The data have failed to identify any association between a unique protease and a specific type of cancer, suggesting that tumour cells take advantage of the many proteolytic enzymes naturally available. Interestingly, they have also shed light on important and unique aspects of tumour cell–stromal cell interaction. For example, many published reports have documented the preferential localisation of proteases in the adjacent stromal cells rather than in invasive malignant cells, suggesting that tumour cells can trigger the production of proteases by surrounding stromal cells [41,44–46]. The recent partial characterisation of a tumour cell-derived factor that stimulates the production of collagenase, stromelysin and gelatinase A by normal cells supports this concept [47]. The identification of specific membrane-bound receptors for uPA [21], plasminogen/plasmin [23], and putatively gelatinase A [35] at the surface of many tumour cells also suggests the existence of another mechanism of interaction. Via these receptors, tumour cells can concentrate soluble proteases produced by stromal cells on their plasma membrane at the cell–matrix interface. Furthermore, activation of these membrane-bound proteases can specifically occur at the surface of tumour cells [48]. Receptor-bound plasminogen can be efficiently activated by receptor-bound uPA located in close proximity [23, 49], and a MT-MMP can activate membrane-bound progelatinase A [30]. Proteases also closely interact at the level of the substrate. For example, in human melanoma cells that secrete large amounts of uPA and interstitial collagenase, removal of glycoproteins by uPA was found to be a prerequisite and rate-limiting step for the degradation of interstitial collagen [50]. These observations indicate that invasive cancer cells have many genuine ways to degrade the ECM (Figure 1). They have the ability to increase their proteolytic activity without increasing their own production and secretion of proteases, and can concentrate and activate proteases in the pericellular space. By producing a variety of proteases, they can also achieve optimal matrix degradation. Thus, attempts to specifically control the production and secretion of proteases in tumour cells will be hampered by the presence of these routes of escape, and therefore inhibition of membrane-bound activated proteases or of binding of pro-enzymes to membrane associated receptors may represent important alternative ways to inhibit tumour cell proteolysis [48, 51]. To target inhibition to more than one family of proteinases may also be essential.

PROTEINASE INHIBITORS

Proteinase inhibitors are classified into physiological inhibitors naturally present in tissues and non-physiological inhibitors that are either produced by micro-organisms or chemically synthesised. Natural (endogenous) inhibitors appear always to be proteins and are important tools used by nature to limit the effect of activated proteases, whereas inhibitors produced by micro-organisms are small non-proteinaceous inhibitors that impair the proteolytic activity of the host. Natural inhibitors have the following potential advantages: they can be produced in a recombinant form, their activity can be modified by site directed mutagenesis, and their expression in cells can be altered by genetic manipulation. Alternatively, synthetic inhibitors are small compounds that can be engineered and modified with an increasing degree of accuracy as the three dimensional structure of many proteases, and their active site is now unveiled by X-ray crystallographic studies. A discussion of all these inhibitors is outside the scope of this article which will focus on natural protein proteinase inhibitors and on some synthetic inhibitors of particular interest in cancer.

Natural inhibitors of serine proteinases belong to the superfamily of serpins (*ser*ine *p*roteinase *in*hibitors) that includes among more than 40 members, α_1-antitrypsin, ovalbumin (without inhibitory function), antithrombin III, PAI-1 and PAI-2, protease nexin-1, and the recently described maspin [52, 53]. Serpins are large single chain (glyco)proteins comprising about 400 amino acid residues that interact with serine proteinases in a unique way [8]. The three dimensional structure of many serpins, including ovalbumin, α_1-antitrypsin and α_1-antichymotrypsin, has been reported, and for PAI-1, crystallographic data of the latent (inactive) form is known [54]. These data have shown that 80% of the amino acid residues of serpins are contained within secondary elements such as α-helices and β sheets, and have pointed to the presence of a unique canonical binding loop that provides an interactive site with the enzyme. Based on X-ray crystallographic information, kinetics data and

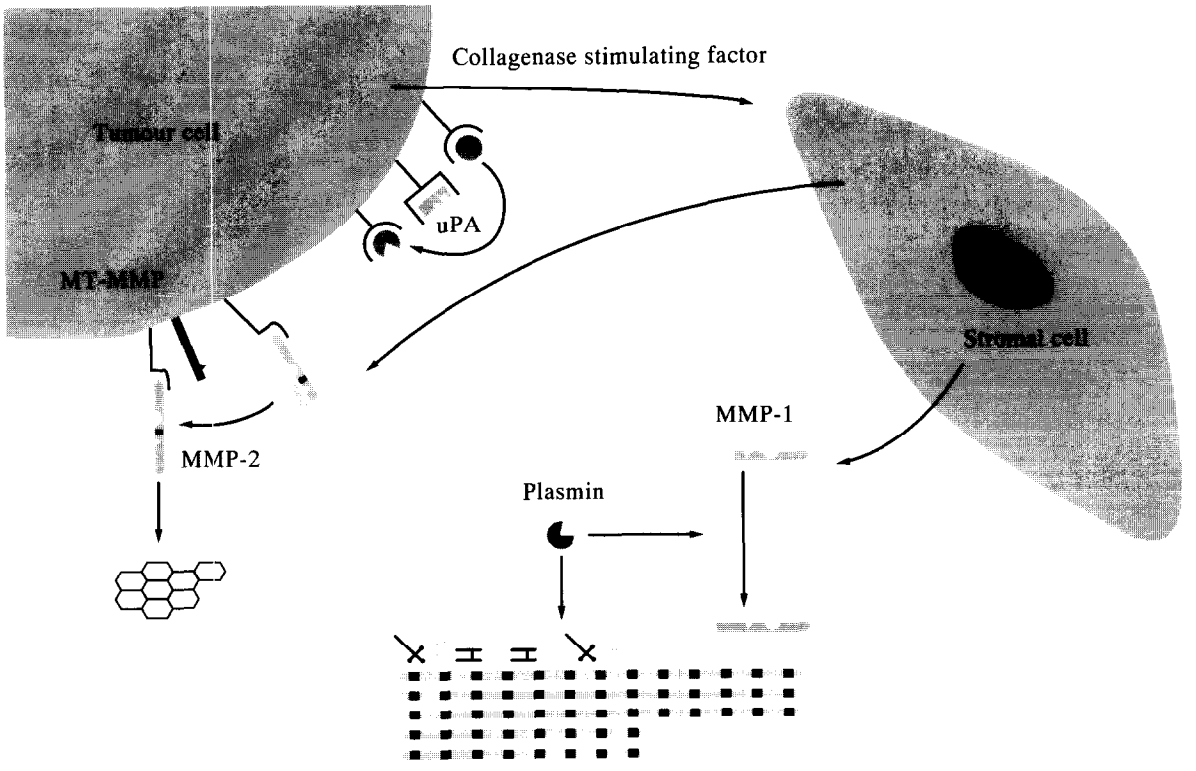

Figure 1. Optimal use of proteases by cancer cells. Schematic representation of the interactive aspects between cancer cells and stromal cells, and serine proteases and matrix metalloproteinases (MMPs), illustrating the importance of pericellular proteolytic activity in cancer cells. Tumour cells secrete "factors" that enhance the production of pro-MMPs by stromal cells. The presence of urokinase type plasminogen activators (uPA) receptors on the surface of tumour cells allows for the concentration of uPA, and uPA mediated activation of receptor-bound plasminogen at the cell suface. The presence of a membrane-type MMP (MT-MMP) and a putative receptor for progelatinase A similarly results in preferential activation of this pro-MMP at the surface of tumour cells. Other non membrane-bound pro-MMPs can be activated in the tumour pericellular space by receptor-bound plasmin. Plasmin and MMP-1 co-operate at several levels. Plasmin activates pro-MMP-1 and degrades glycoproteins, exposing interstitial collagen to the proteolytic activity of activated MMP-1. Natural inhibitors of proteases interfere with these processes in many ways. The tissue inhibitor of metalloproteinases type 2 (TIMP-2) prevents the binding and subsequently activation of progelatinase A, and TIMP-1 and TIMP-2 inhibit activation of pro-MMPs by plasmin as well as inhibit active MMPs. Plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) bind to membrane-bound uPA, preventing activation of plasminogen into plasmin and subsequently activation of pro-MMPs. Subsequently, inhibition of plasmin mediated degradation of glycoprotein will prevent the degradation of collagen by activated MMPs.

mutagenesis studies, it has been possible to postulate the presence of two interactive sites between serpins and serine proteinases (Figure 2). The first site consists of a substrate recognition site (S) where the loop interacts in a substrate-like manner with the enzyme. The second site consists of a segment of the inhibitor exposed to the protease active site (A) which is cleaved during the formation of the enzyme–inhibitor complex at a specific scissile bond (P_1–P_1'). This latter site is responsible for the specificity of each serine protease and for differences in the affinity of individual serpins for serine proteases. For example, in antithrombin III, the P_1 residue is Arg, while in α_1-plasmin inhibitor it is Met, corresponding to the specificity of the peptide bond of the two proteinases respectively inhibited (thrombin and elastase). Exchange of these residues results in loss of inhibitory activity. Inhibition of serine proteinases by serpins evokes, therefore, a suicidal process in which formation of an enzyme–inhibitor complex (EI) results in cleavage of the inhibitor and formation of a complex between an enzyme and a cleaved inhibitor (EI*). This complex can further dissociate, releasing an inactive inhibitor and an active enzyme [8].

$$E + I \rightleftarrows EI^* \underset{k^{stab}}{\overset{k^{dis}}{}} E + I^*.$$

When k^{diss} is lower than k^{stab}, the reaction leads to the formation of a stable enzyme–inhibitor complex. If the k^{diss} exceeds the k^{stab}, inhibitor inactivation without significant inhibition of the enzyme becomes the predominant event. Another specific feature of some serpins is the requirement for an added cofactor necessary to either expose the binding loop or maintain its integrity, therefore promoting inhibitory activity. The best example of antithrombin III which interacts rapidly with thrombin in the presence of heparin but is inactive in its absence. The addition of this sulphated glycosaminoglycan causes conformational changes in the inhibitor exposing the binding loop. A similar effect of polysulphonated oligosaccharides has been shown for protease-nexin 1 and PAI-1.

Cystatins and stefins are tight reversibly binding protease inhibitors that specifically inhibit cysteine proteinases. These inhibitors are found in many tissues including lymph nodes, epithelium, spleen, liver and neutrophils. The X-ray crystal structures of two representative inhibitors, chicken egg white cystatin and stefin B complexed with papain, have been reported [54]. Cystatins/stefins consist of a long central α-helix wrapped in a five-stranded antiparallel β-pleated sheet. This structure exposes two β-hairpin loops that bind to the surface of the enzyme at areas (L) adjacent to the catalytic residues (Figure 2). In a third binding site, the NH_2 terminal domain of the inhibitor interacts with the substrate binding site of the enzyme (S). The structure of the complex results in a rigid amino-terminal trunk that remains uncleaved at the active (cysteine) site.

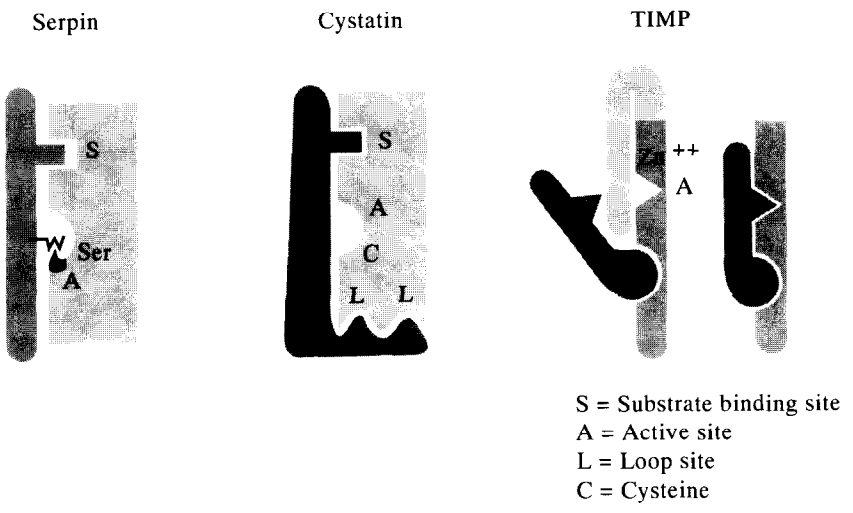

Figure 2. Protease–protease inhibitor complexes. (a) Serpin: The canonical binding loop binds to two sites on the enzyme. At the first site, the inhibitor interacts in a substrate/product-like manner with the substrate recognition site of the enzyme (S). At the second site, the loop interacts with the site containing the active serine (Ser) residue (A). This latter interaction results in cleavage of the inhibitor and formation of a stable proteolytically modified protease–protease inhibitor complex. Adapted from W. Bode and R. Huber, based on X-ray crystallographic information of the trypsin–ovomucoid complex [54]. (b) Cystatin: The inhibitor binds via two hairpin loops (L) to two sites located on the surface of the enzyme adjacent to the reactive site (A). The NH_2 terminal portion of the inhibitor also binds to the substrate recognition site of the enzyme (S) via residues 8 and 9 in a substrate-like manner, but only lies over the reactive site (A) containing the active cysteine residue (C) preventing proteolytic cleavage of the inhibitor. Adapted from W. Bode and R. Huber, based on X-ray crystallographic information on the papain–cystatin complex [54]. (c) TIMP–MMP complex. The inhibitor binds via its C-terminal domain, to the haemopexin (vitronectin-like) domain of the MMP. Activation of the MMP and proteolytic cleavage of the prodomain exposes the catalytic domain (a) and allows for the interaction with the N-terminal portion of the inhibitor. This model is based on mutagenesis and tryptic mapping experiments and not on X-ray crystallographic information. The exact sites of interaction and binding are, therefore, not currently known.

Naturally occurring inhibitors of MMPs belong to a specific class of inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) [55, 56]. Three members of this family, TIMP-1, 2 and 3, have been described so far in humans. Although these three inhibitors are active against most members of the MMP family, they differ in their solubility (TIMP-1 and TIMP-2 are found in soluble forms, whereas TIMP-3 tightly binds to ECM proteins), regulation and ability to specifically interact with pro-MMPs. TIMPs inhibit active MMPs by forming with the activated enzyme tight 1:1 stoichiometric ($Ki \sim 10^{-9}$M) non-covalent complexes that are resistant to denaturation and proteolytic degradation. In addition to inhibiting the activated enzyme, TIMPs also control the autocatalytic activation of many MMPs [57–60] and have the ability to form complexes with pro-enzymes (Figure 2). TIMP-1 and TIMP-2 form complexes stable in sodium dodecyl sulphate with interstitial procollagenase [57], TIMP-1 forms preferential complexes with progelatinase B [61], and TIMP-2 with progelatinase A [62]. Interestingly, these TIMP–proenzyme complexes retain inhibitory activity for activated enzymes, suggesting, therefore, the presence of at least two distinct enzyme–inhibitor binding sites [63–65]. Although crystals of a truncated TIMP-2 ($\Delta_{128-194}$) and an unglycosylated form of TIMP-1 have been obtained [66, 67], the three-dimensional structure of the TIMPs remains currently unresolved. However, based on the assignment of the disulphide bonds that link the 12 cysteine residues placed in preserved positions in the three TIMPs, it is possible to envisage TIMPs as made of two globular domains, each encompassing three interlinked disulphide bonds. Tryptic mapping and mutagenesis studies have suggested that the NH_2 terminal domain of TIMP interacts with the Zn^{++} binding domain of MMP whereas the C-terminal domain binds to the vitronectin-like domain and acts as a stabilisation site [63, 65, 68, 69].

Strategies to design synthetic inhibitors of proteases have taken advantage of our knowledge of the structure of the substrate binding site on the enzyme, the active catalytic site of the enzyme and the structure of natural inhibitors. Thiol-based peptides with IC50 in the 10–20 nM range that inhibit collagenase by mimicking the collagen binding site have been produced [70], and similar peptides that mimic the binding structure of cystatin C have been shown to inhibit cysteine proteinases [71]. A large number of small derivatives of hydroxamic acid have also been synthesised. These are powerful inhibitors of proteases with many different mechanisms of action. Among these, derivatives that specifically inhibit MMPs have been the subject of increased interest [72, 73]. One of these synthetic compounds, BB-94 (Batimastat), binds with very high affinity ($Kd \geq 10^{-11}$ and IC50 in the 1.5–50 nM range) to MMPs by occupying the Zn^{++} binding pocket of the enzyme. Chemically modified tetracyclines have also been proposed as inhibitors of MMPs, although their mechanism of action is less well known. These inhibitors either inhibit the production of MMPs or act as chelators [74–77]. Many of these synthetic compounds and their derivatives are stable, of low molecular weight and are active at a concentration of the nM range that is achievable in tissues. A therapeutic role for these inhibitors in arthritis [75, 76], periodontal disease [77] and cancer [78, 79] has been suggested. They are likely to become powerful therapeutic tools.

THE PROTEASE–PROTEASE INHIBITOR BALANCE IN CELLULAR INVASION

Proteolytic degradation of the ECM and cellular invasion are not unique to malignant cells, and are an essential aspect of many physiological processes such as embryogenesis, organogenesis, trophoblastic implantation and neovascularisation. During angi-

ogenesis, for example, endothelial cells secrete proteases including PAs and MMPs to penetrate the ECM and to form neovascular structures [18]. Similarly, during embryonal implantation, trophoblastic cells secrete proteases to penetrate and anchor into maternal tissues [80–82]. However, in these processes, the proteolytic degradation of the ECM is limited and remains controlled by the coordinated presence of natural inhibitors. In contrast, during malignant invasion, the protease–protease inhibitor balance appears altered resulting in excessive degradation of the ECM [17]. In support of this concept, investigators have demonstrated that disruption of the protease–protease inhibitor balance in several physiological processes results in changes in cell behaviour that closely mimic those seen in malignant cells. For example, overexpression of an autoactivated form of stromelysin-1 under the control of a mammary gland specific gene promoter in transgenic mice results in loss of basement membrane integrity, supernumerary branching of the primary ducts, hyperproliferation of acini cells and reduction in mammary specific function [83]. Alternatively, downregulation of TIMP-1 in mouse NIH 3T3 fibroblasts by the antisense approach renders these cells tumorigenic, invasive and metastatic [84], and embryonic stem cells in which the TIMP-1 gene has been knocked out have been shown to become more invasive *in vitro* [85].

As expected, overexpression of proteases in tumour cells enhance their invasive and metastatic potential. For example, transfection of sense cDNA for uPA and tPA in H-ras-transformed NIH-3T3 cell promotes invasion *in vitro* and metastasis *in vivo* in cells that overexpress these proteases [86], and overexpression of uPA receptor in human osteosarcoma cells also increases matrix invasion [87]. Overexpression of recombinant matrilysin in prostate carcinoma cells transfected with a matrilysin cDNA enhances their ability to invade through the peritoneal cavity [88], and transfection of gelatinase B cDNA in non-metastatic oncogene-transformed fibroblasts confers metastatic capacity to clones overexpressing the MMP [89]. Conversely, overexpression of inhibitors of proteases in tumour cells has been shown to dramatically inhibit malignant behaviour. Overexpression of PAI-2 in human HT1080 cells inhibits their invasive behaviour, and results in the formation of encapsulated tumours *in vivo* [90]. Human melanoma cells transfected with PAI-2 cDNA and overexpressing PAI-2 show a markedly decreased ability to form spontaneous metastasis in scid mice [91], and overexpression of maspin in breast carcinoma cells limits invasion of a reconstituted basement membrane [52]. Khokha and associates have reported that mouse B16-F10 melanoma cells genetically engineered to overexpress TIMP-1 are less invasive *in vitro* [92], and express a lower metastatic potential in chick embryo [93] and in mice [94]. In our laboratory, we have similarly demonstrated that overexpression of TIMP-2 in c-Ha-Ras transfected rat embryo cells suppresses their invasive behaviour in nude mice and partially prevents lung colonisation *in vivo* [95].

Interestingly, these studies have also demonstrated that inhibitors of proteases are potent inhibitors of tumour growth *in vivo*. Overexpression of TIMP-1 in genetically engineered B16 melanoma cells resulted in a substantial decline in tumour growth characterised by a reduced tumour incidence and longer periods before the appearance of tumours [92]. Furthermore, overexpression of TIMP-1 in these cells was found not to reduce their ability to extravasate, but rather to affect tumour growth postextravasation [96]. Similarly, we have shown that overexpression of TIMP-2 in c-Ha-Ras transfected rat embryo cells [95] and human melanoma cells [97] inhibits tumour growth rate in mice. Although the reasons for the growth inhibitory effect of protease inhibitors are presently unclear, several (non-mutally exclusive) possibilities exist. First, many protease inhibitors have been shown to inhibit angiogenesis [98, 99], and this antiangiogenic activity is likely to significantly limit the growth rate *in vivo*. Second, since the ECM is a reservoir of many matrix-bound growth factors [100], it is conceivable that by maintaining the integrity of the ECM, proteinase inhibitors exert an important control of the bioavailability of these factors that could limit the growth of tumour cells. In support of this latter possibility, we have shown that human melanoma cells transfected with TIMP-2 cDNA and selected for TIMP-2 overexpression, grew at a much slower rate when injected subcutaneously in scid mice than parent cells or mock transfectants [97]. *In vitro*, no difference in growth rate was observed when the cells were maintained on regular plastic or gelatin coated tissue culture dishes, however, a significant growth inhibitory effect was documented when TIMP-2 overproducing cells were plated in a three-dimensional collagen gel. Consistent with the concept that the growth inhibitory effect of TIMPs is mediated via the ECM, rTIMP-2 did not inhibit the growth of parent cell lines *in vitro* when cells were plated on gelatin coated dishes, but inhibited growth and promoted differentiation of cells plated in a three-dimensional collagen gel [97].

Thus, it appears that the activity of proteinase inhibitors is not restricted to inhibition of invasion and metastasis, and that by maintaining the integrity of the ECM, protease inhibitors preserve the delicate balance that exists between tumour cells, matrix-bound growth factors and cytokines, and matrix components. Consequently, protease inhibitors may have a marked cytostatic effect on tumours. Altogether, these observations indicate that the balance between proteases and their inhibitors, rather than overexpression of proteases, is a key determinant in tumour progression. Restoration of the imbalance by inhibitors can have a marked suppressive effect on tumour growth, invasion and metastasis.

PRECLINICAL STUDIES

Several physiological as well as non-physiological inhibitors of proteases have been tested in a large number of tumour models *in vitro* and *in vivo*. A summary of the most relevant studies is provided in Table 2. *In vitro*, protease inhibitors have been shown to block the proteolytic degradation of ECM proteins by tumour cells and to inhibit invasion of artificial tissue substrates, such as reconstituted basement membrane (Matrigel), rat smooth muscle cell matrices (SMC), human amnion membranes and chicken chorioallantoid membrane (CAM) [101–111]. Most studies have shown an inhibitory effect of many inhibitors including serpins, cathepsin inhibitors and matrix metalloproteinase inhibitors. Some studies have also pointed to important co-operative aspects between members of different protease inhibitor families [50, 112].

In several of these studies, inhibitors have been shown to inhibit lung colonisation (experimental metastasis) and haematogenous spread (spontaneous metastasis) of tumour cells. rTIMP-1 [107, 113] and rTIMP-2 [114] inhibited lung colonisation of tumour cells injected in the tail vein of mice. Synthetic inhibitors of collagenase [110, 115], serine proteinases [111] and cathepsins [116] have a similar inhibitory activity on the development of blood borne metastasis in ovarian, colon and bladder cancer cells. Using spontaneous and orthotopic metastatic models, other investigators have shown that inhibitors of

Table 2. Preclinical studies of proteinase inhibitors in cancer

Class of proteinases	Natural	Cells and experimental model	Synthetic	Cells and experimental model
1. Serine proteinases	rPAI-1	Fibrosarcoma, colon ca; SMC [101]	FOY 305	Squamous cell ca; inhibition of growth *in vivo* [119]
	rPAI-2	Human melanoma; SMC [50]		
			TAPP-Br	Colon ca; inhibition of growth *in vitro* [117]
	Protease nexin-1	HT1080; SMC [102]		
			Nafamostat mesylate (FUT 175)	Colon ca; exp met [111]
			4-substituted benzo [b]thiophene-2 carboxamidines	HT1080; fibronectin degradation [120]
2. Cysteine proteinases	Cystatin C	Colon ca; matrigel [103]	$ZPhePheCHN_2$ $ZPheAlaCH_2F$	B16, murine mammary ca; amnion [104]
	$ZPhePheCHN_2TPId$	B16, murine mammary ca; amnion [104]		
	$ZPheAlaCH_2FTPIn$		E-64	HOC-I ovarian ca; matrigel [105] Human bladder ca; matrigel, exp met [116]
3. Aspartic proteinases	Pepstatin	Breast ca; ECM degradation *in vitro* [106]		
4. Metalloproteinases	rTIMP-1	B16; amnion and exp met [107] c-Ha-ras transfected cells; SMC and exp met [113]	SC44463	M2, B16; exp met [110] HT1080; matrigel [110]
			BE 16627B	HT1080; growth *in vivo* and exp met [115]
	rTIMP-2	c-Ha-ras transfected cells; SMC [108]		
		HT1080; matrigel [109]	Batimastat (BB-94)	Ovarian ca; spont met [78] Colon ca; spont met [79]
			Chemically modified tetracyclines (minocycline)	B16; tumour-induced red blood lysis [74]

SMC, smooth muscle matrices; exp met, experimental metastasis; spont met, spontaneous metastasis; ca, carcinoma.

proteases are also active inhibitors of local tumour invasion. For example, Davies and associates examined the effect of BB-94 (Batimastat) on human ovarian carcinoma xenografts growing in nude mice. Treatment of animals with daily intraperitoneal injections of 40 mg/kg of BB-94 caused a resolution of the ascitic disease, normally seen after implantation of tumour cells in the peritoneal cavity. Tumour cells became surrounded by a capsule of host cells and formed avascular tumours loosely attached to the peritoneal cavity and often necrotic [78]. This effect was associated with an improvement in the survival of animals in the treatment group. It is interesting to note that many of these studies also pointed to growth inhibitory activity of protease inhibitors [78, 79, 115]. In some cases, a direct effect on cell division has been suggested. For example, Nishimura and associates reported that the serine protease inhibitor TAPP-Br inhibits the growth of human colon carcinoma cells *in vitro* [117]. This effect was associated with downregulation of many oncogenes and growth factors, such as *MYC, FOS, JUN*, TGF-β and EGF that classically have a phorbol and serum responsive elements in their promoter. Murphy and collagues reported on the inhibition of b-FGF growth stimulation of endothelial cells by TIMP-2 [118]. Other experiments performed *in vivo* have suggested an indirect effect of protease inhibitors on tumour growth that involves growth regulatory factors and the ECM. Ohkoshi and associates have shown that a serine protease inhibitor FOY-305, inhibits, in the presence of heparin, the growth of 3-methylcholanthrene-induced squamous cell carcinoma in mice [119]. The authors speculated that FOY-305 prevents the proteolytic digestion of cell surface molecules that restrain proliferation. Third, inhibition of angiogenesis by protease inhibitors may have a significant negative effect on tumour growth [98, 99]. These studies are consistent with the suggestion that the balance between proteases and protease inhibitors is an important factor in tumour progression. Furthermore, the inhibitory effect on the growth of solid tumours has important implications for the possible therapeutic use of these inhibitors.

CLINICAL APPLICATION OF PROTEASE INHIBITORS

For many years, prevention of cancer cell dissemination has been the main potential target for protease inhibitors in cancer. However, this possible application is limited, since the great majority of patients at the time a cancer is diagnosed already have visible or invisible (microscopic) metastases. It was also proposed that these inhibitors could prevent further dissemination of tumour cells at the time of surgical resection. Although of potential value, this approach would require the ability to detect circulating tumour cells by sensitive techniques that are not yet routinely available. For these particular reasons, the idea that protease inhibitors could be used in the treatment of human cancer was justly met with scepticism.

However, as our understanding of the biological activity of protease inhibitors has increased, and as their inhibitory role in angiogenesis and tumour growth has now been demonstrated,

novel therapeutic roles are being suggested. Protease inhibitors could have a significant cystostatic activity on a primary tumour and on established metastatic lesions. Their ability to block the proteolytic activity in tumours could stimulate the stromal content and encapsulation of highly invasive and unresectable tumours. This effect could then allow a later resection that would preserve important normal organ structures located in the vicinity of the tumour. The growth inhibitory activity seen with many of these inhibitors *in vivo* also suggests that they could stabilise or induce regression of primary tumours, and established metastatic lesions that are resistant to cytotoxic agents. Finally, as inhibitors of angiogenesis, protease inhibitors may reduce tumour cell intravasation and prevent the growth of micrometastatic lesions that require an angiogenic response for macroscopic development. Used in this way, it is possible that these inhibitors could be used as adjunctive agents to complement cytotoxic therapies.

However, these approaches may require prolonged administration of these inhibitors which may lead to unacceptable toxicity. Because of their involvement in blood coagulation, serine protease inhibitors will be particularly difficult to administer systemically. Inhibition of matrix metalloproteinases, which are involved in many physiological functions, such as tissue repair and reproduction, could be associated with significant toxicity. Therefore, targeting these inhibitors more specifically to tumour tissues may become an important issue. Synthetic inhibitors may have to be linked to target vehicles such as tumour-specific monoclonal antibodies. The expression of natural inhibitors could be genetically manipulated in tumour cells by target delivering cDNA using a variety of vectors such as liposomes, adenovirus or retrovirus currently tested in some gene therapy protocols. Because the activity of protease inhibitors seems essentially mediated by the ECM, it may not be necessary to deliver and express the gene in every single tumour cells as it would be required for a cytotoxic gene. Expression of these genes in a small proportion of tumoral cells (malignant and non-malignant) may result in the production of enough inhibitor to reverse the protease–protease inhibitor imbalance and maintain the integrity of the ECM.

CONCLUSION

In summary, understanding of the role of proteases and their inhibitors in tumour progression has reached a critical step. From initial correlative evidence linking proteases to invasive and metastatic behaviour of tumour cell lines, we have now obtained direct proof that proteases play an active role in the progression of human cancer. We have become aware of important interactions between tumour cells and host cells, and of genuine mechanisms used by malignant cells to take advantage of stromal cell-derived proteases, and to concentrate proteolytic activity at the cell–matrix interface. Our knowledge of the mechanisms that control protease activity has also dramatically progressed. Many natural protease inhibitors have been identified and cloned, and the three-dimensional structure of several of them and of their complexes with inhibitors have now been revealed by X-ray crystallographic data, allowing the design and development of inhibitors with increased potency and selectivity. It is also becoming evident that, by their ability to preserve the integrity of the ECM, protease inhibitors are more than simple inhibitors of invasion and metastasis, and have a profound inhibitory effect on tumour growth and angiogenesis. Finally, as we realise that cytotoxicity may not always be achievable in disseminated cancers, the concept of using cytostatic agents alone or in combination with cytotoxic drugs may become an attractive and acceptable form of cancer treatment.

The new body of knowledge about the molecular and cellular role of proteases and their inhibitors is now making it possible to carefully conceive clinical trials in which protease inhibitors are used [121], and one such trial has been recently initiated in ovarian cancer [15]. However, since cytostatic anticancer therapy will require chronic administration to achieve a meaningful antitumour effect, systemic toxicity to normal tissues will remain an important issue. Progress in drug delivery and genetic manipulation may be necessary in order to deliver these inhibitors more selectively to tumour tissues and avoid unacceptable long-term side effects. It is hoped that, in the future, protease inhibitors will become part of the therapeutic tools available to treat cancer.

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