

Proteases and Protease Inhibitors: Implications in Antitumorogenesis and Drug Development

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ABSTRACT The role of proteases in cancer is far more complex than initially anticipated and include tumor promoting as well as suppressive effects and their inhibitors are emerging with promising therapeutics in cancer treatment. Proteases are involved in tumor growth both at the primary and metastatic sites. Inhibitors of all the five classes of proteases (serine, aspartyl, cysteine, metallo- and threonine) have been widely reported from plant, animal and microbial origin. Each protease exhibits a characteristics "recognition-specificity" and are specific to cleave proteins with a particular structure. Such a capability allows identifying signatures of protease activity in biological fluids. Individual pattern of protease expression help in easy prognosis and therapeutic administration of specific protease inhibitor. With the advance of surface enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry, proteomic technologies are directly applied into clinical diagnostic tests. The substrate phage technology can be used to develop protease profile signatures for all type of cancers. Recently, application of biomarkers in cancer treatment has become increasingly target-oriented. Gene expression signatures in cancer provide molecular phenotype that identifies tumor classification not evident by traditional histo-pathological methods. The current review deals with the role of proteases, their inhibitors in cancer especially in tumor progression, invasion/metastasis and also addresses the therapeutic approaches, viable strategy for cancer treatment and regime of the future drug development.

INTRODUCTION

Recent studies have shown that proteases are involved in progression, tumor growth both at the primary and metastatic sites. Extracellular proteases may co-operatively influence matrix degradation and tumor cell invasion through proteolytic cascade with individual protease having distinct roles in tumor growth, invasion, migration and angiogenesis (Koblinski et al. 2000). Proteases (PRs) are expressed in the extracellular milieu as inactive proforms that are activated through a variety of mechanisms, which often involve a close collaboration among several families of PRs (Coussens et al. 2002).

There is a positive correlation between the aggressiveness of a tumor and the secretion of various PRs. Approximately 500-600 proteases have been known to exist in human and mouse genome but not all of them have been found to be linked with cancer (Puente et al. 2003). Multiple alterations in a normal cell can lead to a localized tumor and finally to one that has the ability to invade and metastasize. Tumor cell invasion involves attachment of tumor cells to the underlying basement membrane, local proteolysis and migration of tumor cells through

its modified region (Liotta et al. 1983). PRs, which are expressed in these cells, are believed to participate in many of these steps (Fig. 1).

PRs such as aspartic, cysteine, serine and metalloproteinases are crucial in cancer propagation and inhibitors of such enzymes are emerging with promising therapeutic uses. Aspartic proteases such as cathepsin D and E, matrix metalloproteinases (MMPs) and serine proteases have been studied the most, and they affect tumor progression in many stages.

Epigenetic changes that occur in normal epithelial cells (NEC) lead to tumor formation and growth. Tumor cells (TC) also undergo epithelial-mesenchymal transformation during the same time. Formation of neovessel is stimulated where endothelial cells (EC) proliferate and invade towards the tumor site. TC also invades the connective tissue and then intravasate. The TC must arrive in circulation, arrest, extravasate, invade the local environment and grow to set up distant metastasis. These metastasis steps occur through the interactions of TC, EC, fibroblasts and invading inflammatory cells (IC), such as macrophages and the extracellular matrix. Steps where PRs are believed to participate in this process are shown in solid arrow.

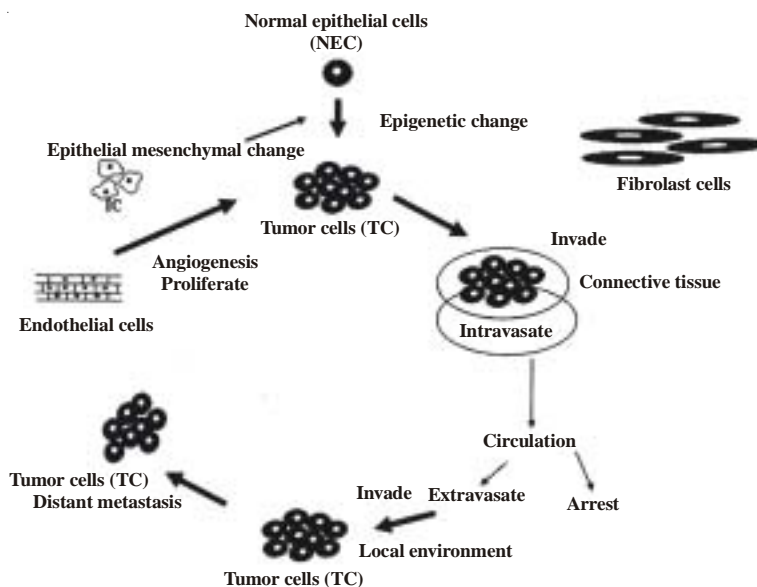


Fig. 1. The role of PRs in the metastatic process

Recently, developed functional genomic approaches, such as DNA microarrays have enabled researchers to determine the expression level of every gene in a given cell or cancer population, which represents that cell population's entire transcriptome. cDNA array allows us to define specific molecular pathways of tumoral progression and to define markers of prognostic and diagnostic relevance which reflects significantly on cancer diagnostics and consequently its management. They also boost our knowledge of the molecular events responsible for the development and progression of cancer (Polyak and Riggins 2001).

Proteases Involved in Tumor Invasion and Metastasis of Cancer

PRs, which promote or directly contribute to the degradation and remodeling of the basement membrane and extracellular matrix are considered most important in metastasis and angiogenesis (Bernstein and Liotta 1994). Cysteine protease such as cathepsin B, L, K, Q, S and aspartic proteases-cathepsin D and E are mainly involved in intracellular proteolysis within lysosomes. Plasmin urokinase type plasminogen activator belongs to serine protease and gelatinase A (MMP2) and B (MMP9) of matrix metallopro-

teases are the other proteases responsible for extracellular proteolysis. Threonine protease (proteasomes), has the task of eliminating cellular proteins, tagged for degradation through a complex modification termed polyubiquitination. It is a process of addition of a series of ubiquitin molecules to another protein, targeted for degradation (Mitchell, 2003). Proteases families involved in tumor invasion and metastasis are given in Table 1.

Cathepsin D is one of the most widely studied acid PRs in cancer research, it not only facilitates tumor progression but may have a prognostic value in patients with breast cancer. Human cathepsin D is an intracellular aspartic proteinase mainly found in lysosomes. It has a number of house keeping functions including degradation of cellular or phagocytosed proteins for reprocessing. Chemokines are a family of structurally related glycoproteins with potent leukocyte activator and/or has chemotactic activity but very limited information is available on the interaction between chemokines and PRs (Vicari 2002).

Cathepsin D is known to degrade chemokines and consequently affect their putative functions in the tumors (Wolf et al. 2003). Investigators are targeting the overexpression of PRs in tumors by developing novel clinical prodrugs, which are

inactive until activated by PRs. Cathepsin E is neither secretory nor lysosomal but it is located in endoplasmic reticulum/ trans-Golgi network/ endosomal compartments of cells (Kageyama 1998). Aoki et al. (1995), reported conversion of cathepsin E (CE) to enzymatic unstable form i.e. medium moving proteinase (Med.P) in gastric cancer cells and Med.P is a monomeric form of this protease (CE).CE is also expressed in pancreatic cancer (Azuma et al. 1995).

Cathepsin L has been implicated in tumor metastasis, the process where tumor cells detach from the primary lesion and migrate through lymph or blood vessels to form new foci at distant sites (Navab et al. 1997). Cathepsin B was the first lysosomal protease to be associated with breast carcinoma (Poole et al. 1978). Procathepsin B (proCB) gets activated by tissue plasminogen activator (tPA), which results in activation of plasmin and MMPs which leads to degradation of components of extracellular matrix. Procathepsin B (proCB) may be activated by tPA initiating a proteolytic cascade which results in the activation of plasmin and MMPs. Collectively, active proteases can degrade all components of the extracellular matrix (Fig. 2). The involvement of cathepsin in regulation of angiogenesis reveals

Table 1: Proteases involved in tumor invasion and metastasis

<i>Metalloproteinases</i>	Matrix metalloproteinases, collagenases (MMP-1,-8 and -13), gelatinase (MMP-2,-9), matrilysins (MMP-7,-26, MT- MMPs (MMP 14,-17,-24,-26, stromelysin (MMP-3,-10,-11), other MMPs (MMPs-12,-19,- 20, 23- 27 and -28) and A Disintegrin And Metalloprotease (ADAMS)
<i>Serine proteinase</i>	Cathepsin G, chymase, chymotrypsin, membrane bound serine proteases, elastase, plasmin, plasminogen activators, trypsin, tryptase and Human tissue Kallikreins.
<i>Other proteinase</i>	Aspartic proteases (cathepsin D and E), cysteine proteases (cathepsin B,H,K,L,M,N,Q, O, S), caspases (bleomycin hydrolases) and threonine proteases (proteasome)

another distinct role in tumor progression (Joyce et al. 2004).

Increased expression, activity and changes in localization including altered sub-cellular distribution, surface localization and secretion of cathepsin B have been observed in colorectal (Campo et al. 1994), gastric (Watanabe et al.

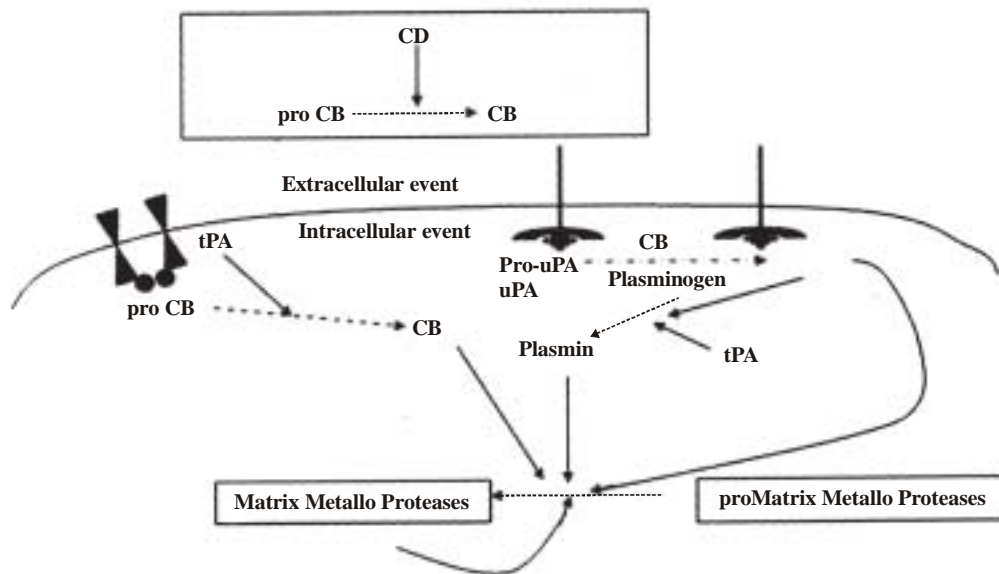


Fig. 2. Cascade of proteolytic activation

1989), lungs (Sukoh et al. 1994) prostate (Sinha et al. 1995), glioma (Rempel et al. 1994) melanomas (Rozhin et al. 1994) and oestoclastomas (Page et al. 1992), suggesting that PRs might be involved in the development, invasion and metastasis of more than one type of tumor. Local extracellular-matrix degradation by proteolytic enzymes is an important feature of tumor invasion and metastasis of malignant solid tumors (Fidler, 1990). Plasmin and urokinase-type plasminogen activator (uPA) play a central role in cancer cell invasion (Mignatti and Rifkin 1993). Trypsinogens are serine PRs that play a significant role in tumor progression. The production of tumor-associated trypsinogen (TAT-2) has been correlated with malignant phenotype (Koivunen, et al. 1990). The expression of many of the membrane anchored serine proteases is widely dys-regulated during tumor growth and progression. The first membrane anchored serine protease discovered is enteropeptidase (enterokinase) and matriptase3 is the one of the most novel ones (Netzl-Arnett et al. 2003).

Eighteen zinc containing endoproteinases, referred to as matrix metalloproteinases (MMPs), are collectively capable of degrading collagen, proteoglycan and produced by variety of cells (Goetzl et al. 1996; Cawston 1995). It plays an important role in normal tissue remodeling and may also contribute to the local growth of tumors and the development of metastasis because of the role they play in tumor cell invasion and angiogenesis (De Clerck et al. 1994). Also, these enzymes have been associated with the progression of diseases in a number of malignancies, including breast, colorectal, gastric, pancreatic and non small cell lung (NSCL) cancers (Basset et al. 1993; Hewitt et al. 1991; Ray and Stetler-Stevenson 1994). At present, 24 different vertebrate MMPs have been identified, 23 of which have been found in humans (Visse and Nagase, 2003). The involvement of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) is well documented in breast, ovarian, colorectal and lung tumors (Brown and Giavazzi 1995).

Masson et al. (1998) suggested that MMPs are involved in early alterations leading to tumor formation. Mice deficient in stromelysin-3 exhibit lower tumor incidence and tumor size after carcinogen treatment. Over expression of some MMPs leads to development of pre-malignant and pre-neoplastic lesions (Sympson et al. 1995), suggesting that MMPs participate in different

stages of tumor progression prior to invasion and metastasis (Wilson et al. 1997). They are known to increase cell proliferation by activating growth factors or liberating them from the ECM where they are sequestered. Growth factors like bFGF, EGF, IGF, TGF- β and VEGF are bound to ECM and can be released upon proteolysis of the extracellular matrix (ECM) components (Taipale and Keski-Oja 1997; Whitelock et al. 1996). Noel et al. (1993) showed that the ability of fibroblasts to promote tumorigenicity of MCF7 cells requires matrigel containing low molecular weight factors. MMP inhibitors abolish the tumor promoting effects of the fibroblasts, suggesting that MMPs from the fibroblasts release growth factors from the matrigel.

A family of membrane anchored PRs called 'A Disintegrin And Metallo-protease (ADAM) proteins have emerged as the major proteinase family that mediates ectodomain shedding, the proteolytic release of extracellular domains from their membrane bound precursors. ADAMs bind to integrins, inhibit tissue inhibitor metallo-proteinase (TIMP) activity and affect intracellular signaling through ectodomain shedding (Huovila et al. 2005). ADAMs are proteins that contain both a disintegrin and metalloproteases domain and have potential implications for the metastasis of human cancer cells via cell adhesion and protease activity. ADAM-17/TACE is the best characterized molecule of the ADAMs family, since it is involved in the proteolytic cleavage of the soluble form of tumor necrosis factor α (TNF- α) (Karan et al. 2003).

Proteasomes and human tissue kallikreins (hks) are the recent emerging targets for therapeutics. The ubiquitin-proteasome pathway is responsible for most eukaryotic intracellular protein digestion. This pathway has been validated as a target for antineoplastic therapy using both *in vitro* and preclinical models of human malignancies and is influenced as part of the mechanism of action of certain chemotherapeutic agents (Orlowski and Claire Dees 2003). The proteasome is responsible for the degradation of all short-lived proteins and 70-90% of all long-lived proteins (Pajonke and McBride 2001). The hks are primarily known for their clinical applicability as cancer biomarkers, recent evidences indicates hks in many cancer related processes, including cell growth regulation, angiogenesis, invasion and metastasis. They have been shown to promote or inhibit neoplastic progression, acting individually and /

or in cascades with other hks and other proteases and might represent attractive targets for therapeutic interventions (Borgono and Diamandis 2004). Prostate-specific antigen (PSA, hk3) and human glandular kallikreins (hk2) are widely used tumor markers for prostate cancer. Three other kallikreins, hk6, hk10 and hk11, are emerging new serum biomarkers for ovarian, prostate cancer diagnosis and prognosis (Diamandis and Yousef 2002).

Protease Inhibitors in Antitumorogenesis

The five major classes of protease enzymes selectively catalyze the hydrolysis of polypeptide bonds and are crucial for disease propagation and inhibitors of such proteases are emerging with promising therapeutic uses in the treatment of diseases like cancers. Protease inhibitors can suppress several stages of carcinogenesis, including initiation, promotion and progression, although their mechanism of action is not yet fully clear. Inhibitors of aspartic peptidases are relatively uncommon and are found in only a few specialized locations (Bennette et al. 2000). Few of the examples include a 17kDa inhibitor of pepsin and Cathepsin E from the parasite *Ascaris lumbricoides* (Kageyama 1998; Ng et al. 2000), proteins from plants such as potato, tomato and squash (Christeller et al. 1998) and a pluripotent inhibitor from sea anemone of cysteine peptidase as well as cathepsin D (Lenarcic and Turk 1999) (Table 2).

BBI, the soybean derived PRs inhibitor is a potent chymotrypsin inhibitor that has been extensively studied as this is the PRs inhibitor that has risen to the human trial stage as a human

cancer chemopreventive agent (Fernandes and Banerji 1997). Pepstatin, a low –molecular weight aspartic peptidase inhibitor, isolated from various sp. of *Streptomyces* , is a specific inhibitor of pepsin (Umezawa et al.1970) and cathepsin D belong to this class of aspartic peptidase inhibitors. Relatively few inhibitors of cathepsin D have been reported, partly because of its uncertain role as a viable target for therapeutic intervention.

Tolcher et al. (2001) discussed the current status of PRs inhibitors as cancer chemo-preventive agents. They report on the present evaluation, both preclinical and clinical, of the intervention of soy PRs inhibitors that reduce superoxide-induced DNA damage in prostate cancer prevention. A large number of PRs inhibitors have been isolated from potatoes and related plants. The potato PRs inhibitors that have been purified and extensively characterized fall into three main categories. Inhibitor I, II and carboxypeptidase inhibitor. Potato I inhibitor family is also referred to as chymotrypsin inhibitor I, because its specificity is directed most strongly towards chymotrypsin, although it inhibits subtilisin, pronase as well as some other alkaline microbial PRs and it is also a weak inhibitor of trypsin. Trypsins are widely expressed in various tissues and cancer cells (Kato et al. 1998). The potato carboxypeptidase inhibitor is an epidermal growth factor (EGF) antagonist that inhibits tumor cell growth (Blanco-Aparicio et al.1998). Aprotinin has been shown to control plasmin activity involved in tumor-cell spreading and to prevent tumor invasion (Verstraete 1985).

Peptidic inhibitors of great interest are epoxysuccinyl derivatives such as E-64, isolated

Table 2: Protease inhibitors implicated in cancer.

<i>Inhibitor</i>	<i>Source</i>	<i>Enzyme</i>	<i>Disease</i>	<i>Reference</i>
17kDa inhibitor	<i>A. lumbricoides</i>	Pepsin Cathepsin E	Cancer	Kageyama (1998)
Pluripotent	Sea anemone	Cathepsin D	Cancer	Lenarcic and Turk 1999
Bortezomib (PS-341)	Synthetic	Proteasome	Prostatic cancer	Voorhees et al. 2003
Bowman–Birk inhibitor (BBI)	Soybean	Cathepsin D	Gastric cancer	Fernandes and Banerji 1997
BMS 275291	Synthetic	MMPs 1,2,,8,9,13, 14 and 3	Lung cancer	Shepherd (2001)
AE-941 (Neovastat)	Shark cartilage extract	MMP-2, MMP-12	NSCLC	Gingras et al. 2004
BB2516 (Marismastat)	Synthetic	MMP-1, 2, 7, 8, 9	Breast cancer NSCLC, glioma	Summers and Davidsen, 1998
MR889	Synthetic	Neutrophil elastase	NSCLC	Luisetti (1996)
Col-3, CMT-3 (Metastat)	Synthetic	Gelatinase (MMP-2,9)	Glioblastoma, Kaposi’s sarcoma	Acharya (2004)

from cultures of *Aspergillus japonicus* that alkylate the active site residue of cysteine proteases. E-64 is a broad-spectrum inhibitor, which also effectively inhibits cathepsin L and calpain. This inhibitor supports development of cathepsin L inhibitors as potential therapeutics as antimetastatic agents (Leung et al. 2000).

The development of small molecular weight inhibitors of MMPs offered the potential of preventing tumor invasion and angiogenesis thus inhibiting tumor growth and metastasis. Interference with the activity of MMPs through the expression of endogenous tissue inhibitors of MMPs (TIMPs) has been shown to inhibit invasion *in vitro* and *in vivo* and can block tumor-induced neovascularization (Murphy et al. 1994). The use of novel, non-classical anticancer agents such as MMPs inhibitors represent a new and potentially effective approach. AG3340 (Prinomastat) is targeted against MMP1 with specificity directed against MMP2 and MMP-9 and this agent has been evaluated in two trials of patients with stage IIIB and IV NSCLC (Shepherd 2001).

BAY 12-9566 (Tanomastat) is a MMPs inhibitor that has pre-clinical activity in ovarian cancer models. It is a novel and specific non-peptidic biphenyl MMP inhibitor against several MMPs implicated in tumor progression (Rowinsky et al. 2000). BMS-275291, an orally bio-available MMP inhibitor currently under development, has been shown to decrease the overall tumor burden in several animal models (Poulaki 2002). It is the newest MMP1 to be evaluated in lung cancer. It is broader spectrum with activity against MMPs 1, 2, 8, 9, 13, 14 and to some extent MMP3. MMP inhibitors (MMPI) are primarily considered to have anti-proliferative rather than direct cytotoxic effects. Neovastat (AE-941) is a shark cartilage extract, which has

been shown to have MMP inhibitor activity against MMP-2 and MMP-12. Clinical benefits observed upon Neovastat treatment rely on the presence of multiple angiogenesis inhibitors including inhibitors of MMP activities (Gingras et al. 2001).

Neovastat specifically stimulates tPA-dependent plasmin generation through an increase in the affinity of the enzyme towards plasminogen apart from its stimulatory effect on tPA activity, neovastat also markedly stimulates tPA expression in endothelial cells through an increase in the transcription of the tPA gene (Gingras et al. 2004). Anginex is a potent inhibitor of EC adhesion and migration and function by apoptosis (Griffioen et al. 2001).

Marimastat is a broad-spectrum MMP inhibitor. It is currently in phase III clinical trials and is being evaluated for the treatment of invasive cancers and metastasis (Summers and Davidsen, 1998) (Fig. 3A). Recent advances in development of tetracycline derivatives as potential inhibitors of MMPs, which have shown promising preclinical and early clinical results (Acharya et al., 2004). BILA 2157BS is another potent rennin inhibitor with some selectivity towards cathepsin D (Simoneau et al. 1999). Brem et al. (1990) found that a mild penicillamine-induced copper deficiency greatly reduced the growth of the tumors and their invasiveness. The cyclic thiol MR889 has been investigated as a chemotherapeutic agent for lung cancer (Inada et al. 1997) (Fig. 3B). With its low toxicity and good *in vivo* properties, it may soon enter human trials. Bortezomib (PS-341), proteasome inhibitor, (Fig. 3C) is the first inhibitor to undergo clinical testing, has demonstrated impressive antitumor activity and manageable toxicities in phase I and II trials both as a single agent and in combination with other drugs (Voorhees et al. 2003). Inhibitors

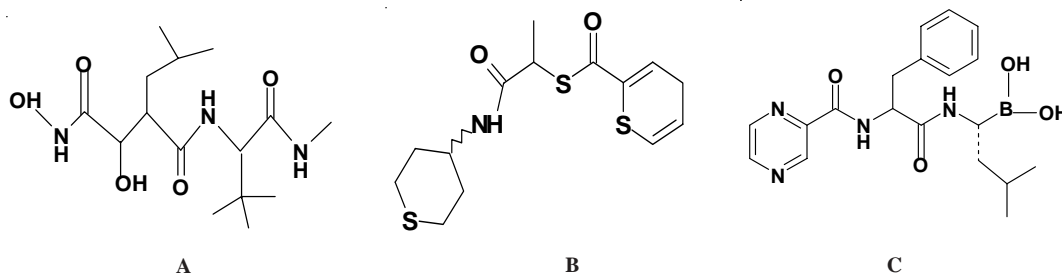


Fig. 3. Chemical structure of potent protease inhibitors implicated in antitumorigenesis: A: Marimastat. B: MR889. C: Bortezomib (PS- 341).

of the proteasome impact on cells, in part, through down regulation of nuclear factor κ B, but also through modulation of cell cycle proteins and other pro and anti apoptotic pathways. The development of specific and potent chemical inhibitors of the proteasome has sparked considerable excitement about the therapeutic potential of this class of drugs not only in cancer but other immune disorders (Mitchell 2003). MG-132, vinyl sulfone, epoxomicin, lactacystin and clasto-lactacystin b-lactone are some of the representative classes of proteasome inhibitors.

ADAM activities are regulated by a group of physiological inhibitors called (TIMPs). TIMPs have shown the inhibiting properties against the protease activity of certain ADAM molecules. TIMP-1 and TIMP-3 has been shown to inhibit the protease activity of ADAM-10, while only TIMP-3 has been demonstrated to be inhibitory towards ADAM-17/TACE (Amour et al. 1998).

Genomics and Proteomics of Proteases in Tumorigenesis

Cancer is a genetic disease caused by changes or modification of DNA sequences of key genes that result in altered gene or protein expression or altered protein composition of tumor cells. Genetic changes and environmental factors interact to influence cancer development (Rasooly and Jacobson 2006). It is now possible with new genomic and proteomic technology using single nucleotide polymorphism, proteomics, epitomics and messenger ribonucleic acid analysis, molecular approaches to early detection and prognostic determination in prostate cancer possible. Through out the world, technologies in molecular diagnostics and prodiagnostics are being developed that are enhancing our ability to gain insight into molecular targeted agents (Rashid 2006).

Genomic structural changes have been deduced under certain pathological conditions using a variety of methods such as Comparative Genomic Hybridization (CGH), analysis for Loss of Heterozygosity (LOH) and Fluorescence *in situ* Hybridization (FISH). Several chromosomal regions have been identified for their frequent deletions in various types of cancer. Chromosomal deletion is an early and frequent somatic genetic alteration during carcinogenesis. Gene expression patterns resulting from regional chromosomal events such as gene amplification,

deletion, rearrangement and epigenetic transcriptional mechanisms are obscured in patterns derived from remote loci. Hierarchical clustering, K-means clustering, self-organizing maps and support vector machines are some commonly used methods in microarray data analysis (Yi, 2005). VEGF mRNA expression in lung cancer, tyrosine kinases in leukemia and HER-2 receptors in breast cancer are well-characterized targets. All of these targets have been identified in late invasive and metastatic cancers, thereby limiting the success of treatment. However, if targets in the earliest stage of the tumor can be identified, then treatment is likely to be more successful. Recently, application of biomarkers in cancer treatment has become increasingly target oriented (Negm 2002).

Human tissue kallikreins, (hK3) also known as prostate-specific antigens (PSA) is primarily known their clinical applicability as cancer biomarkers. These are secreted serine PRs and are the latest arrival on the cancer proteolysis scenario. Evidence indicates the importance of kallikrein in many cancer related process, including role in PRs activation cascades, cell growth regulation, angiogenesis, invasion and metastasis (Borgono and Diamandis, 2004). With the improvement in proteomics technology, it is now possible to adopt these technologies directly in clinical diagnostic tests such as SELDI-TOF mass spectrometry. A discriminatory spectrum proteomics profiles could be generated using this technology with a pattern recognition based bioinformatics tool, that would help distinguish men with prostate cancer from those with benign prostates. This methodology will improve the specificity and sensitivity of prostate cancer after technological difficulties are overcome and refinements are made in this technique (Ornstein 2006). Earlier, most proteomics studies have used the tumor tissue itself as the source for biomarker discovery but with the advances in proteomics technologies and the knowledge of the molecular milieu of even localized cancer is reflected in circulating fluids.

From differential analysis for the identification of biomarkers to functional analysis for the identification and/or validation of new therapeutic targets, proteomics brings new comprehensive information for a better understanding of the molecular basis of oncology and new perspectives for the clinic. However, the major limitation of proteomics investigations and more generally

of post-genomic approaches remains the molecular and cellular complexity of biological systems. Traditionally, proteomics studies have been used for biomarker discovery and the clinical test is typically an enzyme-linked immunosorbent assay (ELISA) based assay. Furthermore, with the advent of high-throughput methods, it is possible that some of these analytical instruments will become usable for proteomic-based clinical diagnostics.

This is in contrast to a common belief that activity of PRs mostly has a negative influence in cancer. With the discovery of efficient and specific inhibitors, it was supposed to be an efficient cancer treatment. Relatively benign cancers acquire malignant properties when PRs expression is up regulated. Highly malignant cells become less aggressive when PRs expression or activity is reduced. Many angiogenesis inhibitors are stored as cryptic fragments within larger precursor matrix molecules that are not themselves antiangiogenic and the regulation of proteolytic processing plays an important role in the vascularization of tumors. It contributes more to tumor progression and regulation than just by degrading physical ECM barriers and enabling cell migration and tumor invasion. They release matrix-bound growth factors and endogenous angiogenesis inhibitors, process anti-inflammatory chemokine, expose cryptic integrin-binding sites and affect cell-to-cell interactions and apoptosis. Promoter methylation has been well recognized as an important epigenetic change in the development of cancer (Baylin and Herman 2000).

Gene Expression Signatures in Cancer

Traditional methods of phenotypic characterization are often limited and do not have the ability to discern subtle differences that may be of importance for developing a better understanding of the tumor and advancing therapeutic strategies for the treatment of disease. A gene expression database contains the expression profile of 218 tumor samples representing 14 common human cancer classes. Accurate multi class classification is now possible, making molecular cancer diagnosis feasible by means of comparisons with a comprehensive and commonly accessible catalogue of genes expression profile (Ramaswamy et al. 2001). A variety of biochemical and molecular factors that

are reported to have prognostic or predictive ability include cathepsin D, HER2, EGFR, p53, UPA, PAI etc. Human squamous cell carcinomas (SCC) are characterized by up-regulation of MMP1, 10, 13 and cathepsin L2 (Haider et al. 2006). MMP-2 and MMP-9 have been shown to be up regulated and that the two forms have a significant role in many of the pathologic conditions related to metastasized cancers associated with tumor aggressiveness, metastatic potential and poor prognosis (Johansson et al. 2000). TIMPs have been associated with less aggressive and favorable prognosis in patients. However, other studies have shown increased expression of TIMPs, particularly TIMP-2, to correlate with more aggressive tumor development (Karameris et al. 1997). Thus TIMPs may have a dual effect on tumor growth and metastasis and in addition to suppressing proteolysis and neovascularization of MMP. They also may promote tumor cell proliferation during discrete stages of tumor development (Henriet et al. 1999). Chemically modified tetracyclines (CMTs) have also been shown to down regulate expression of gelatinases and thus to reduce the production of proenzyme. Also, CMTs inhibit the activation of collagenase and gelatinase pro enzyme form (Golub et al. 1991).

Gene expression information generated by DNA microarray analysis of human tumors can provide molecular phenotyping that identifies distinct tumor classifications not evident by traditional histopathological methods. The promise of such information lies in the potential to inform and so improve clinical decisions and strategies used to treat patients with neoplastic disease (Alizadeh et al. 2000). The Ovarian Cancer Prognostic Profile (OCP), a 115-gene signature is an independent prognostic determinant of outcome in epithelial ovarian cancer (EOC). The use of gene profiling may ultimately permit identification of EOC patients appropriate for investigational treatment approaches, based on a low likelihood of achieving prolonged survival with standard first line platinum based therapy (Spentzos et al. 2004).

Enzyme based histochemical analysis showed that some leukemia's were periodic acid-Schiff positive, whereas others were myeloperoxidase positive. This provides the first basis for classification of acute leukemias into those arising from lymphoid precursors (acute lymphoblastic leukemia, ALL) from myeloid precursors (acute

myeloid leukemia, AML) (Golub et al. 1999). The use of expression profiling with cDNA array techniques in mammary tumor cell lines and breast tumors help in classifying controversial tumors and provide new prognostic tools and potential therapeutic targets (Bertucci et al. 2001). Numerous studies have correlated genetic alterations with clinical outcome including a strong correlation between the amplification of the erbB-2 receptor gene (Her-2) and poor clinical outcome (Ciocca et al. 1992). Also over expression of erbB-2 is a strong predictor of response to adriamycin- based therapy (Muss et al. 1994). The analysis of gene expression represents an indirect measure of the genetic alterations in tumor because these alterations affect gene regulatory pathways. The analysis of gene expression represents an indirect measure of the genetic alterations in tumors because in most instances these alterations affect gene regulatory pathways. The tremendous complexities that can be scored by measuring gene expression with DNA microarrays together with the absence of bias in assumptions as to what type of pathway might be affected in a particular tumor, the analysis of gene expression profile offers the potential to impact clinical decision-making based on more precise determination of tumor cell phenotypes (West et al. 2001).

Although cancer classification has improved over last 30 years, there has been no general approach for identifying new cancer classes (class discovery) or for assigning tumors to known classes (class prediction). The feasibility of its classification based solely on gene expression monitoring and suggests a general strategy for discovering and predicting cancer classes for other types of cancer, independent of previous biological knowledge. Improvements in cancer classification have thus been central to advances in cancer treatment. Tumors with similar histopathological appearance can follow significantly different clinical courses and show different responses to therapy (Golub et al. 1999).

Classification of leukemias and lymphomas that have been achieved in recent year's analysis of gene expression patterns represent significant steps in the development of methodologies to phenotype tumors (Golub et al. 1999). Bayesian regression models that provide predictive capability based on gene expression data derived from DNA microarray analysis of a series of primary breast cancer samples, have the capacity

to discriminate breast tumors on the basis of estrogen receptor status and also on the categorized lymph node status (West et al. 2001).

Using the gene expression profiling patterns patients were classified into two groups namely, the St. Gallen criteria and National Institute of health (NIH) consensus criteria. The St. Gallen and NIH criteria classify patients as at low risk or high risk on the basis of various histological and clinical characteristics. This comparison shows that the prognosis profile assigned many more patients with lymph node-negative diseases to the low risk (good-prognosis signature) group than did the traditional methods (Van de Vijver et al. 2002). The primary breast tumors were classified as having either a poor prognosis signature, which means they were likely to metastasize, or a good-prognosis signature, meaning that the development of metastasize was unlikely. The classification of patients in to high risk and low risk subgroup on the basis of the prognosis profile may be a useful means of guiding adjuvant therapy in patients with lymph node- positive breast cancer. This approach also improves the selection of patients who would benefit from adjuvant systemic treatment, reducing the rate of both over treatment and under treatment. Other methods of classification are based on OVA SVM-based and S2N classifier.

Perou et al. (1999) identified tumors with distinct patterns of gene expression that they termed basal and luminal type. Microarray analysis has been used to distinguish cancers associated with BRCA1 or BRCA2 mutations (van't Veer et al. 2002) to determine estrogen-receptor status (Gruvberger et al. 2001) and lymph node status (Ahr et al. 2002). Fulvestrant is the pure estrogen antagonist, which help in degradation of estrogen receptor α . It works by enhancing proteasome-dependent degradation of the client protein via ubiquitin-proteasome pathway. It is approved for use by postmenopausal patient with estrogen receptor positive breast cancer who has progressed following other anti-estrogen therapy (Orlowski and Claire Dees 2002). The three-gene expression predictive of subsequent relapse status comprised genes involved in cell cycle control (CCNE1), DNA methylation (DNMT3B) and DNA damage repair (BRCA2). This gene expression signature is an introductory candidate for routine clinical use, especially as the three genes encode well characterized protein for which specific anti-

bodies are already available (Bieche et al. 2004). hK8 is the favorable prognostic marker in ovarian cancer (Borgono et al. 2006). PAI and uPA are independent prognostic markers in breast cancer. Apart from the lymph node status, high levels of uPA and PAI-1 are the strongest prognostic markers for disease free survival and overall survival. Patients with lymph node – negative tumors the levels of uPA and PAI-1 are the strongest predictor of metastasis. In addition, the two markers might also be used to predict response therapy in particular the response to adjuvant therapy (Weigelt et al. 2005). ONCOMINE is a database that connects the cancer microarray database to several sources of additional information, including the scientific literature, the human protein reference database and online catalog of all proven disease-gene connections.

Drug Design and Development

Numerous practical applications in the area of cancer research and the understanding that PRs are important targets for the drug design, ultimately fuelled much research in this field. Specific PRs inhibitors, likely in combination with conventional anticancer agent will probably prove to have value for certain forms of cancer. The substrates identified for PRs provide a “signature” set of peptides that can be used to identify the activity of these PRs in blood. These substrates can be incorporated into diagnostic assays, or into imaging agents to image the position of distant metastasis, or to monitor disease progression. In addition, the substrate phage technology can be pushed to a level that is likely to help reveal the substrate recognition specificity of all PRs in human genome. This technology can also be used to develop PRs profile signatures for all types of cancers (Kridel et al. 2001).

Differences in the sub-site structure of human cathepsin D and related human aspartic proteases may be exploited for drug design (Baldwin et al. 1993). It is now becoming obvious that cysteines and cathepsins should be seriously considered as potential target in cancer treatment. Serine PRs TAT-2 (Tumor associated trypsinogen) might be a good candidate for a clinical prognostic marker that could identify patients with an aggressive disease (Nyberg et al. 2006). XIAP, an endogenous inhibitor of caspases (another family of cysteinyl PRs), could

be an attractive target for cancer drug development, since caspase 3 derepression leads to apoptosis and/or sensitization of tumor cells for chemotherapeutic drugs (Schimmer et al. 2004).

Among all pharmacological targets, inhibition of function of MMPs in ECM after secretion is being most actively pursued as a potential anticancer strategy. Once secreted into the ECM, activation of pro MMP could be inhibited or enzymatic activity could also be directly inhibited (Hindalgo and Echaradt 2001). Matrilysin (MMP7) has been the point of attention because of its preferential expression in early stages tumors and pre-malignant lesions, which make it suitable target for chemo-preventive strategies (Wilson et al. 1997). However, the failure of broad-spectrum MMP inhibitions in clinical trials has opened the door for other PRs to be considered as relevant drug targets in anti cancer therapy. The compound JPM-OEt had profound effects on tumor growth, invasiveness and angiogenic switching, disrupting both early and late stages of tumorigenesis in contrast to MMP inhibitors, which have not proved effective in the later stages of tumorigenesis. A number of PRs, such as proteasome histone deacetylase are emerging as potential drug targets in cancer and now new selective inhibitors of MMPs are being developed (Coussens 2002).

Measuring the “recognition-specificity” for a large number of PRs at one time using a powerful biochemical technique called substrate phage display, millions of different peptide structures (called library) can be displayed on a common scaffold. This library of substrates when exposed to individual PRs, few peptides that are cleaved by the PRs could be identified. Using this strategy unique recognition for three PRs associated with breast cancer, MMP-2, 9, along with membrane type-1 MMPs. Each of these enzymes are involved in tumor invasion, tumor metastasis and perhaps more significantly with tumor angiogenesis. The substrate- based drug design has been substantially improved in recent years the availability of three-dimensional structure information for peptidases, permit receptor based design. The structural information about the active site of the receptor or (peptidase) and selection of designed molecules with the aid of computers has helped to design receptor-based inhibitors. Combinatorial chemistry also presents opportunities both to discover new molecular entities for assaying and to optimize lead struc-

tures for the development of peptidase inhibitors (Leung et al. 2000). A new class of peptidomimetics, the unsymmetrical peptidyl ureas, have emerged as powerful inhibitors of aspartic peptidase (Dales et al. 2001). These were developed using mechanism- based and substrate- based design techniques and using the computational method GrowMol (Ripka et al. 2001).

In *vitro* and in *vivo* studies have shown that PRs inhibitors reduce tumorigenicity. The wide expanse of knowledge gained so far still leaves several key questions unanswered. Analysis of multiple PRs in a single model system would further enhance the elucidation of the function of PRs both in normal physiological conditions and in tumor environment. This will help in the design of better PRs based drugs and inhibitors to combat malignancy (Griffioen et al. 2001).

The ubiquitin-proteasome pathway is just beginning to be exploited as a target for cancer therapy. Bortezomib, most notable proteasome inhibitor is currently being evaluated in clinical trials and has already been found to have significant antitumor efficacy. Its primary action involves modulation of proteasome-ubiquitin activity. Geldanamycin, camptothecins, irinotecan and fulvestrant are some of the drugs that act by increasing ubiquitin-proteasome pathway-mediated degradation of a target protein (Orlowski and Claire Dees 2002).

Several kallikreins are differentially expressed at both mRNA and protein levels in various endocrine-related malignancies and have prognostic value. In addition to their diagnostic/prognostic potential, kallikreins may also emerge as attractive targets for therapeutics (Diamandis and Yousef 2002). Today, the design of PRs inhibitors involves a powerful combination of all these traditional drug discovery approaches, supplemented by *de novo* drug design, combinatorial chemistry and phage display techniques and supported by rapid robotic assay methods to find or optimize inhibitor leads from vast chemical libraries. The next decade will see identification of many new PRs as targets for inhibitor development following the advancements made in the areas of molecular and cellular biology, protein chemistry, microbiology, structural biology, nano biotechnology and molecular pharmacology. The complex roles of PRs in different stages of tumor growth should be kept in mind when designing PRs inhibitors as cancer treatment (Nyberg 2006).

SCOPE OF THE REVIEW AND FUTURE PROSPECTS

Proteases have been widely studied for their role in tumor growth, invasion and metastasis. However, we still not have answered key questions relating to the use of PRs inhibitors for therapeutic interventions. Collaborations like the PRs consortium that analyses multiple PRs in a single tumor would further our knowledge of how PRs are involved in tumor progression as well as help us to design better inhibitors and novel PRs based drugs for clinical use. The European Cancer Proteases Consortium (EUCPC) proposes multidisciplinary research into innovative approach to cancer therapy and diagnosis based on DEGRADOME. It is complete repertoire of extracellular proteases through which cells regulate their local environment. Extracellular protease remains an attractive target for intervention as for cancer, even if first generation anti – protease drugs were disappointing. PRs inhibitor discovery began with natural product screening and substrate- derived analogue- based drug design and then progressed with incorporation of mechanism-based drug design strategies and more recently has advanced further using computer assisted structure-based inhibitor design using three dimensional structures of PRs determined by X-ray crystallography and NMR spectroscopy.

Some of the questions still to be answered is whether one PR or one class of PRs are more important in specific tumor and at specific steps in tumor progression than another, whether extracellular PRs co-operatively influence matrix degradation and tumor cell invasion through proteolytic cascade, or do individuals PRs have distinct influences on tumor growth, invasion, migration and angiogenesis, whether proteolytic cascade is important, can an upstream PRs be targeted so only one PRs inhibitor is needed and finally whether selective inhibitors be able to decrease tumor growth and metastasis, possibly decreasing the side effects from broad host spectrum PRs inhibitors. In this regard, hks, proteasomes and ADAMs are the three upcoming groups of proteases, which require special attention. In view of the above mentioned proteases, their respective inhibitors, aprotinin, bortezomib (PS-341), TIMP-3 (inhibitor of ADAM-17/TACE demand equal thrust and are the future therapeutic agents in cancer treatment

regime. On the basis of findings, it is now established that a few kallikreins have already found important clinical applications, whereas other members show promising potential. ADAMs is a relatively recent discovery. Therefore, studies on these molecules in tumor cells require further elucidation. In spite of their potential roles in cancer metastasis through metalloproteases and disintegrin domains, the expression studies and hormonal regulation of ADAMs is poorly documented in cancer cell types. Mature phase II data evaluating bortezomib in combination with other agents for solid tumors and trials evaluating its role earlier in multiple myeloma therapy, are eagerly awaited as efficacy of bortezomib in solid tumors are less mature. Researches at the University of Michigan, Johns Hopkins and the Institute of Bioinformatics in India have discovered a gene expression signature common to distinct types of cancer, thus giving new dimensions to the diagnostic and prognostic methods by developing a universal treatment for the world's second most dreaded disease. This is a unique approach in a sense that all earlier approaches invariably focused on the differences in the gene expression pattern of different types of carcinomas. On the basis of illustrations in this review, it does not take much speculation to understand that PRs inhibitors will increasingly become valuable molecular probe for improving our understanding of biological processes as well as commercially valuable drug candidate for treating cancer.

The review will be incomplete without the mention of nano-biotechnology, which is one of the upcoming fields of science in the treatment of cancer. It offers us an unprecedented and paradigm-changing opportunity by offering a wealth of tools, such as new and innovative ways to diagnose and treat cancer. Quantum dots, nanopores, and other devices for detection and treatment may be available for clinical use in 5-10 years. The greatest advantage of the nano-biotechnology would be in potentially developing ways to irradiate cancer cells without harming healthy neighboring cells in addition to its role in the early, minimally invasive method of detection of cancer cells. Though nano-biotechnology is a promising approach in the treatment of cancer, certain legal and ethical issues need to be addressed.

Gene expression profiling by DNA microarrays can be used to accurately diagnose and

molecularly classify tumors, assess their propensity to metastasize and predict responses to combination therapy. Therefore, there is keen interest in defining the gene expression of all human tumors to create a new generation of clinically useful cancer diagnosis. DNA microarray technology is easy to use, yield gene expression measurements for thousands of genes simultaneously. This has made possible the prediction of the metastatic potential of a tumor, which requires the analysis of many different markers at once by analyzing gene expression in a genome wide fashion (microarray-plateform). Aggregate patterns of gene expression (metagenes) are associated with lymph-node status at diagnosis and a 3 year-recurrence risk in breast cancer patients of all ages. Further studies are required to validate metagene classifier for breast cancer recurrence. Gene expression signatures might refine the prognostic classification of cancer allowing researchers to move accurately in identifying patients with metastatic risk than the present conventional prognostic markers. The genes that are deregulated, in the molecularly defined classes with poor prognosis, might also constitute novel targets for therapy.

Recently, tumor transcriptome revealed the predictive and prognostic impact of lysosomal protease inhibitors in NSCLC. This provides the first comprehensive molecular characterization of clinical responsiveness to platinum based chemotherapy (PBC) in NSCLC, potentially representing novel targets for NSCLC therapeutics.

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