Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies

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ABSTRACT Over the last years, the relevance of the matrix metalloproteinase (MMP) family in cancer research has grown considerably. These enzymes were initially associated with the invasive properties of tumour cells, owing to their ability to degrade all major protein components of the extracellular matrix (ECM) and basement membranes. However, further studies have demonstrated the implication of MMPs in early steps of tumour evolution, including stimulation of cell proliferation and modulation of angiogenesis. The establishment of causal relationships between MMP overproduction in tumour or stromal cells and cancer progression has prompted the development of clinical trials with a series of inhibitors designed to block the proteolytic activity of these enzymes. Unfortunately, the results derived from using broad-spectrum MMP inhibitors (MMPIs) for treating patients with advanced cancer have been disappointing in most cases. There are several putative explanations for the lack of success of these MMPIs including the recent finding that some MMPs may play a paradoxical protective role in tumour progression. These observations together with the identification of novel functions for MMPs in early stages of cancer have made necessary a reformulation of MMP inhibition strategies. A better understanding of the functional complexity of this proteolytic system and global approaches to identify the relevant MMPs which must be targeted in each individual cancer patient, will be necessary to clarify whether MMP inhibition may be part of future therapies against cancer.

KEY WORDS: *angiogenesis, metastasis, proteases, degradome.*

Introduction

The ability of cancer cells to invade other tissues and spread to distant organs is an often-fatal characteristic of malignant tumours. Proteolytic enzymes play a fundamental role in cancer progression providing an access for tumour cells to the vascular and lymphatic systems, which support tumour growth and constitute an escape route for further dissemination (Chambers *et al.,* 2002; Mareel and Leroy 2003). The complexity of proteolytic systems operating in human tissues is impressive, as assessed by the finding that more than 500 genes encoding proteases or protease-like proteins are present in the human genome (Puente *et al.,* 2003). However, among all the proteolytic enzymes potentially associated with tumour invasion, the members of the MMP family have reached an outstanding importance due to their ability to cleave virtually any component of the ECM and basement membranes, thereby allowing cancer cells to penetrate and infiltrate the subjacent stromal matrix (Brinckerhoff and Matrisian 2002). Although the mechanistic process of ECM degradation mediated by MMPs had been the focus of many investigations for years, recent studies have shown that the role of MMPs in cancer progression is much more complex than that derived from their direct degradative action on ECM components (Egeblad and Werb 2002; Freije *et al.,* 2003; Hojilla *et al.,* 2003). Growth-factor receptors, cell adhesion molecules, chemokines, cytokines, apoptotic ligands, and angiogenic factors are just some examples of the diversity of substrates targeted by MMPs. The recent characterization of new MMP substrates as well as the generation of genetically modified animal models of gain or loss of MMP function, have demonstrated the relevance of MMP activities in the early stages of cancer development. These observations emphasize the importance of re-evaluating the anti-cancer trials that have been developed to inhibit MMPs (Coussens *et al.,* 2002; Overall and Lopez-Otin 2002; Pavlaki and Zucker 2003). The purpose of this review is to present the current knowledge on the functional complexity of the MMP family and to discuss the implications of this new information for designing improved MMP-inhibition strategies for cancer therapy.

Abbreviations used in this paper: ECM, extracellular matrix; MMP, matrix metalloproteinase; MMPI, MMP inhibitor.

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Structural diversity of MMPs

The availability of the complete human genome sequence has allowed to define the complete set of MMPs produced by human cells. Thus, recent genomic studies have revealed that there are 24 distinct genes encoding members of the MMP family (Puente *et al.,* 2003). Analysis of the structural design of these enzymes has led to a new classification system based on MMP structures rather than on their substrate specificities (Fig. 1). Most of them are organized around a conserved catalytic domain which incorporates a propeptide necessary to maintain enzyme latency, a signal peptide which directs their secretion from the cell, and a C-terminal hemopexin domain which contributes to substrate specificity and to interactions with endogenous inhibitors (Overall 2002). This archetypal MMP design is present in the subgroup of secreted proteases composed of the three human collagenases (MMP-1, MMP-8, and MMP-13), the two stromelysins (MMP-3 and MMP-10), and four additional MMPs with unique structural characteristics (MMP-12, MMP-19, MMP-20, and MMP-27). Besides the archetypal conformation, the two matrilysins (MMP-7 and MMP-26) lack the hemopexin domain (Uria and Lopez-Otin 2000) and the two gelatinases (MMP-2 and MMP-9) incorporate three fibronectin type II modules that provide a compact collagen-binding domain (Morgunova *et al.,* 1999). In addition to these secreted MMPs, there are six membrane-type (MT)-MMPs localized at the cell surface through a C-terminal transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP) or by a glycosylphosphatidylinositol anchor (MT4- and MT6-MMP) (Zucker *et al.,* 2003). The MT-MMPs also have an additional insertion of basic residues between the propeptide and the catalytic domain,

which is cleaved by furin-like serine proteases leading to the intracellular activation of the proenzymes (Thomas 2002; Zucker *et al.,* 2003). This furin-like cleavage site is also present in three secreted MMPs (MMP-11, MMP-21 and MMP-28) that do not fit to any of the previous subgroups and in two unusual transmembrane MMPs, (MMP-23A and MMP-23B), which are anchored through an N-terminal segment and show identical amino acid sequence, despite being encoded by two distinct human genes (Pei *et al.,* 2000; Velasco *et al.,* 1999).

To date, and despite significant advances in x-ray crystallography and nuclear magnetic resonance techniques, human MMP-2 is the only MMP family member whose full-length structure has been solved (Morgunova *et al.,* 1999). In addition, the 3D structures of different domains of a number of MMPs have been determined (Bode 2003; Visse and Nagase 2003) (http://www.rcsb.org/pdb/). Nevertheless, it should be essential to increase the number of structures available for MMPs, to better understand the variety of substrates that these enzymes can target as well as to allow the design of more selective MMP inhibitors (MMPIs).

The biology of MMPs

The evolution of the MMP family to generate this structural diversity likely reflects the number and complexity of biological processes in which these enzymes are involved. The identification of new MMP substrates and the development of genetically modified animal models with gain or loss of MMP function, have demonstrated the relevance of these proteases in multiple physiological processes (Vu and Werb 2000) (Tables 1 and 2).
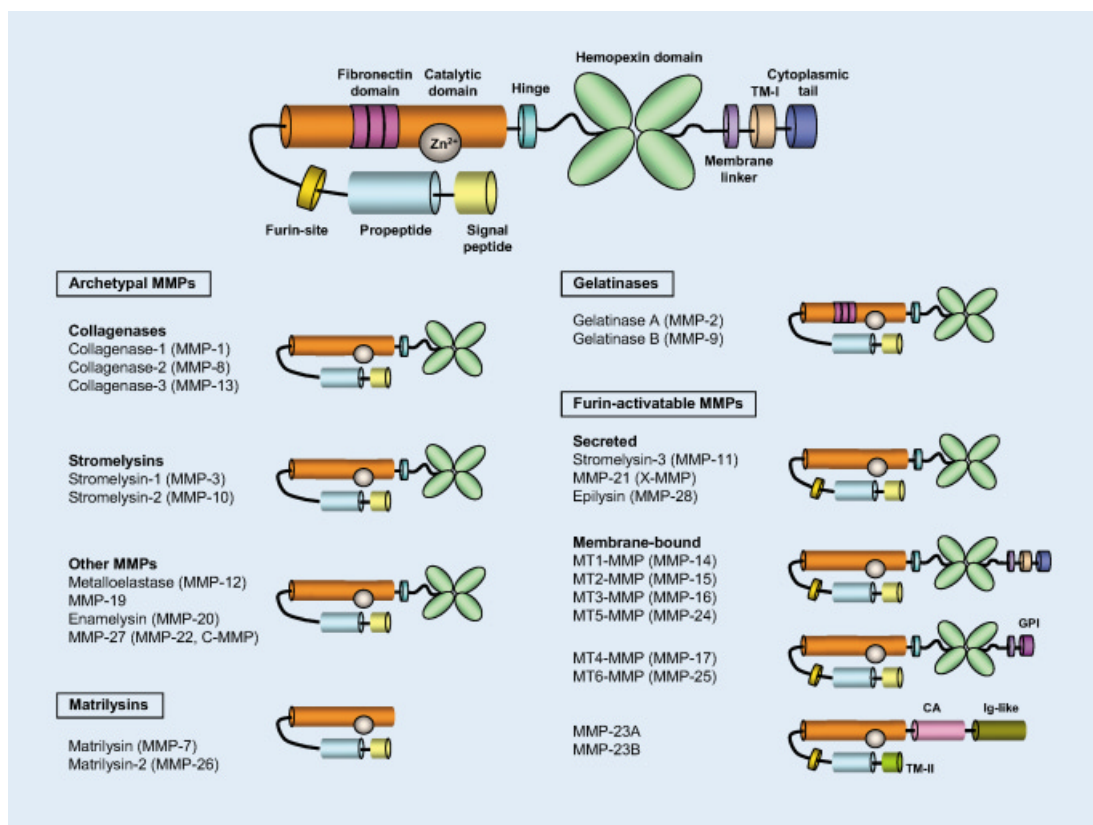

Fig. 1. Diversity of human MMPs. *Structural classification of human MMPs based on their domain organization.*

Physiological functions of MMPs

Embryonic growth and tissue morphogenesis are fundamental events that require disruption of ECM barriers to allow cell migration and matrix microenvironment remodelling. The ability of MMPs to degrade structural components of ECM and basement membranes has supported their direct implication in these processes. In fact, research on the MMP field started with the finding that a collagenolytic activity was responsible for a major developmental event: the tail resorption during metamorphosis in tadpoles (Brinckerhoff and Matrisian 2002; Gross and Lapiere 1962). Nevertheless, the discovery that MMPs are also able to release or process bioactive molecules in addition to their classical degradative properties on structural proteins has provided a new opportunity to appreciate the importance of these enzymes in many biological functions (Vu and Werb 2000).

Most MMP genes are highly expressed in a number of reproductive processes, including menstrual cycle, ovulation, and uterine, breast and prostate involution (Curry and Osteen 2003; Hulboy *et al.*, 1997). Thus, matrilysin, stromelysins and gelatinase A are consistently produced during the most active phases of the murine estrous cycle. These MMPs, as well as collagenase-2 and collagenase-3, are also up-regulated during postpartum uterus involution (Balbin *et al.*, 1998; Rudolph-Owen *et al.*, 1997). In addition, the expression patterns of several MMP genes have been analyzed during gonadotropin-induced ovulation, in order to identify those members responsible for follicular wall degradation (Curry and Osteen 2003; Hagglund *et al.*, 1999). However, none of the mutant mice deficient in specific MMPs which have been generated to date show a significant reproductive dysfunction. This finding suggests that functional redundancy among MMPs, or between these enzymes and components of the plasminogen system may compensate for the loss of a specific MMP (Ny *et al.*, 2002; Solberg *et al.*, 2003).

The relevance of MMPs in embryonic development has prompted the identification and characterization of new members of this family in model organisms such as *Drosophila*, where developmental processes have been extensively studied (Llano *et al.*, 2002; Llano *et al.*, 2000). The discovery that *Drosophila* has only two MMPs has allowed for the first time the complete ablation of all MMPs in any organism, through the creation of a double mutant (Page-McCaw *et al.*, 2003). This study has demonstrated that, in flies, MMPs are required for tissue remodelling but not for embryonic development. However, the importance of mammalian

TABLE 1

SUBSTRATES OF MMPs DISTINCT FROM TYPICAL EXTRACELLULAR MATRIX COMPONENTS

MMP number	Protease name	Bioactive substrates		proMMP activated
MMP-1	Collagenase-1 (interstitial collagenase)	Pro-1L-1β Pro-TNF-α IGFBP-2,-3,-5 SDF-1 MCP-1,-2,-3	L-selectin Perlecan α1-proteinase inhibitor α1-antichymotrypsin α2-macroglobulin	proMMP-1 proMMP-2
MMP-2	Gelatinase A (72 kDa gelatinase)	Pro-1L-1β Pro-TNFα Pro-TGF-β IGFBP-3,-5 FGFR-1 SDF-1	MCP-3 Decorin α1-proteinase inhibitor α2-macroglobulin KiSS-1/metastin Endothelin-1	proMMP-1 proMMP-2 proMMP-13
MMP-3	Stromelysin-1	Pro-1L-1β Pro-TNF-α Pro-HB-EGF IGFBP-3 SDF-1 MCP-1,-2,-3,-4 E-cadherin L-selectin	Perlecan Decorin Endostatin Plasminogen α1-proteinase inhibitor α1-antichymotrypsin α2-macroglobulin antithrombinIII	proMMP-1 proMMP-3 proMMP-7 proMMP-8 proMMP-9 proMMP-13
MMP-7	Matrilysin	Pro-TNF-α Pro-α-defensin Pro-HB-EGF FasL E-cadherin β4 integrin	Decorin Endostatin Plasminogen Syndecan α1-proteinase inhibitor α2-macroglobulin	proMMP-1 proMMP-2 proMMP-7 proMMP-9
MMP-8	Collagenase-2 (neutrophil collagenase)	Pro-TNF-α IGFBP MCP-1 IP-10 MIG	LIX L-selectin α1-proteinase inhibitor α2-macroglobulin α2-antiplasmin	proMMP-8
MMP-9	Gelatinase B (92 kDa gelatinase)	Pro-1L-1β IL-2Rα Pro-IL-8 Pro-TNF-α Pro-TGF-β IFN-β FGFR-1 SDF-1 GROα CTAP-III	IP-10 MIG GCP-2 ENA-78 Tumstatin Endostatin Plasminogen α1-proteinase inhibitor α2-macroglobulin KiSS-1/metastin	proMMP-2 proMMP-9 proMMP-13
MMP-10	Stromelysin-2			proMMP-1 proMMP-8 proMMP-10
MMP-11	Stromelysin-3	IGFBP-1 α1-antitrypsin	α1-proteinase inhibitor α2-macroglobulin	
MMP-12	Metalloelastase (macrophage elastase)	Pro-TNF-α Endostatin	Plasminogen α1-proteinase inhibitor	
MMP-13	Collagenase-3	Pro-TNF-α SDF-1 MCP-3	Endostatin α1-antichymotrypsin α2-macroglobulin	proMMP-9 proMMP-13
MMP-14	MT1-MMP	Pro-TNF-α αvβ3 integrin CD44 SDF-1 MCP-3	Tissue transglutaminase Syndecan α1-proteinase inhibitor α2-macroglobulin KiSS-1/metastin	proMMP-2 proMMP-8 proMMP-13 proMT1-MMP
MMP-15	MT2-MMP	Pro-TNF-α	Tissue transglutaminase	proMMP-2 proMMP-13
MMP-16	MT3-MMP	Pro-TNF-α Syndecan	Tissue transglutaminase KiSS-1/metastin	proMMP-2 proMMP-13
MMP-17	MT4-MMP	Pro-TNF-α		proMMP-2
MMP-24	MT5-MMP	KiSS-1/metastin		proMMP-2 proMMP-13
MMP-25	MT6-MMP (leukolysin)	α1-proteinase inhibitor		proMMP-2 proMMP-9
MMP-26	Matrilysin-2 (endometase)	IGFBP-1	α1-proteinase inhibitor	proMMP-9

MMPs in this process can be appreciated from the early implantation stages, where the production of MMP-9 by invading trophoblasts seems to be critical (Alexander *et al.,* 1996). Furthermore, studies with *Mmp9*-deficient mice have demonstrated the *in vivo* role of this protease in a number of developmental processes. Thus, these mice exhibit a defect in endochondral bone formation, which is accompanied by delayed apoptosis of hypertrophic chondrocytes at the skeletal growth plates and deficient vascularization (Vu *et al.,* 1998). Targeted inactivation of the MT1-MMP gene in mice also causes several skeletal and connective tissue defects, as well as defective angiogenesis, leading to premature death (Holmbeck *et al.,* 1999; Zhou *et al.,* 2000).

The role of MMPs in tissue remodelling has also been demonstrated in several reports. MMP-2 and MMP-3 regulate mammary gland branching morphogenesis during puberty (Wiseman *et al.,* 2003). MMP-2 and MMP-9 also contribute to adipogenesis by promoting adipocyte differentiation (Bouloumie *et al.,* 2001). However, other MMPs seem to have an inhibitory effect in this process. Thus, *Mmp3*-deficient mice show accelerated adipogenesis during mammary gland involution (Alexander *et al.,* 2001). MMPs are also involved in wound healing, a tissue-remodelling process which involves the migration of keratinocytes at the edge of the wound to re-epithelialize the damaged surface. Several studies in cell culture have shown that the proteolytic activity of MMP-1 is required for keratinocyte migration (Pilcher *et al.,* 1997). The *in vivo* role of MMPs in this process has been supported by the analysis of *Mmp3*-deficient mice, which exhibit impaired wound contraction (Bullard *et al.,* 1999), and by studies in collagenase-resistant mice which also show a severe delay in wound healing (Beare *et al.,* 2003). However, the complete inhibition of the healing process requires the blockade of both plasminogen and MMP proteolytic activities, indicating again a functional overlap between both classes of matrix-degrading proteases (Lund *et al.,* 1999).

The role of MMPs in angiogenesis is also wide and complex. Many MMPs are produced by endothelial cells and have been described to be important for the formation of new blood vessels in both physiological and pathological conditions. For example, MMP-2 associates with integrin $\alpha v \beta 3$, and this interaction is essential for localizing the enzyme to the surface of newly forming vessels (Brooks *et al.,* 1994). Further studies examining the links between MMP-2 and angiogenesis have shown that, after different challenges, *Mmp2*-null mice show reduced vascularization compared to wild-type controls (Itoh *et al.,* 1998; Lambert *et al.,* 2003). The finding that choroidal neovascularization is severely impaired in *Mmp2/Mmp9*-double deficient mice has demonstrated the synergic effect of both proteases in this process (Lambert *et al.,* 2003). In addition, enzymatic studies have revealed that the endogenous angiogenic inhibitor endostatin can block the activation or the catalytic activities of MMP-2, MMP-9, MMP-13 and MT1-MMP (Kim *et al.,* 2000; Lee *et al.,* 2002; Nyberg *et al.,* 2003). MMPs may also regulate angiogenesis by acting as pericellular fibrinolysins during the neovascularization process (Hiraoka *et al.,* 1998). Finally, many members of the MMP family show a dual ability to mobilize or activate pro-angiogenic factors or angiogenic inhibitors. The relevance of these MMP functions in cancer will be further discussed in this review.

MMP roles in cancer

The identification of novel biological functions for MMPs has prompted the evaluation of their relevance in cancer beyond the classical MMP roles of ECM disruption in late invasive stages of the disease. Thus, proteolytic processing of bioactive molecules by MMPs contributes to the formation of a complex microenvironment that promotes malignant transformation in early stages of cancer. These additional functions mediated by MMPs include activation of growth factors, suppression of tumour cell apoptosis, destruction of chemokine gradients developed by host immune response, or release of angiogenic factors (Egeblad and Werb 2002; Hojilla *et al.,* 2003) (Fig. 2) (Table 2).

There is an increasing evidence supporting the participation of MMPs in the regulation of tumour growth by favouring the release of cell proliferation factors such as insulin-like growth factors which are bound to specific binding proteins (IGFBPs) (Manes *et al.,* 1997). MMPs may also target and activate growth factors whose precursors are anchored to the cell surface or sequestered in the peritumour ECM (Yu and Stamenkovic 2000). Furthermore, a recent study has illustrated the direct effect of MMP matrix remodelling activity on cell growth (Hotary *et al.,* 2003). This interesting work has shown that the expansion of tumour cells inside a three-dimensional collagen-matrix is significantly enhanced in response to MT1-MMP overexpression. By contrast, overproduction of a number of soluble MMPs did not have any effect on tumour cell growth (Hotary *et al.,* 2003). The ability of MT1-MMP to confer this proliferative advantage to tumour cells is not apparent when cells are placed in a two-dimensional system, confirming
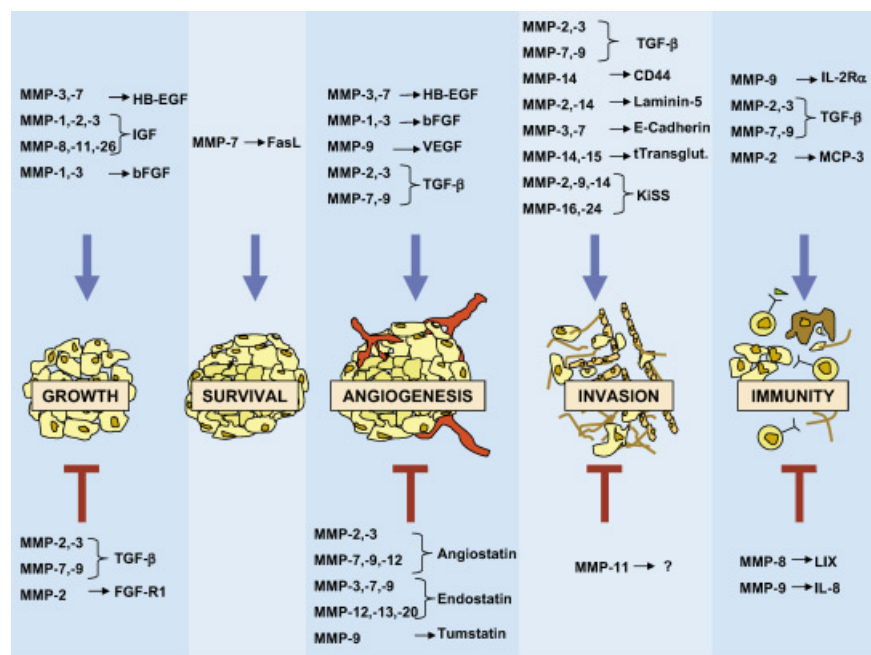
Fig. 2. Dual functions of MMPs in tumour progression. *The opposite effects of bioactive molecule processing by MMPs on cancer development are shown.*

the importance of physical presentation of the surrounding ECM on cell behaviour (Cukierman *et al.,* 2001). Nevertheless, it is remarkable that tumour cells may develop protease-independent migratory mechanisms in response to the blockade of pericellular proteolysis (Wolf *et al.,* 2003).

The ability of MMPs to target substrates that influence the apoptotic process is also relevant for cancer. Thus, MMP-3 has pro-apoptotic actions on the neighbouring epithelial cells (Witty *et al.,* 1995), whereas MMP-7, which is able to release the membrane-bound Fas ligand, also induces epithelial cell apoptosis (Powell *et al.,* 1999). This cleavage can also favour tumour progression as a result of the protection that FasL confers to cancer cells from chemotherapeutic drug cytotoxicity (Mitsiades *et al.,* 2001). Also in this regard, it is of interest that mice deficient in MMP-2, MMP-3 or MMP-9 have lower levels of apoptosis induced by TNF-α, which has suggested that MMPIs may be useful in cancer therapies using inflammatory cytokines (Wielockx *et al.,* 2001). Other MMPs, such as MMP-11, suppress tumour cell apoptosis inhibiting cancer cell death (Boulay *et al.,* 2001). This finding suggests that the targeting of MMP-11 activity could lead to survival benefits for cancer patients. However, a paradoxical effect for this MMP in cancer has been recently described. Thus, *Mmp11*$^{-/-}$ MMTV-ras transgenic mice develop more metastasis than their *Mmp11*$^{+/+}$ MMTV-ras counterparts, despite the lower number and size of primary tumours (Andarawewa *et al.,* 2003). These data imply that in addition to its antiapoptotic action, MMP-11 should have another molecular function that leads to decreased metastatic rate. This observation emphasises the importance of selectively targeting certain MMP functions instead of completely blocking their activity.

MMP activities have also been traditionally associated with a variety of escaping mechanisms that cancer cells develop to avoid host immune response (Coussens *et al.,* 2000; Coussens and Werb 2002). Some MMPs, such as MMP-9, can suppress the proliferation of T lymphocytes through the disruption of the IL-2Rα signalling (Sheu *et al.,* 2001). Likewise, MMP-11 decreases the sensitivity of tumour cells to natural killer cells by generating a bioactive fragment from α1-proteinase inhibitor (Kataoka *et al.,* 1999). In addition, MMPs may modulate antitumour immune reactions through their ability to efficiently cleave several chemokines or regulate their mobilization (Li *et al.,* 2002; McQuibban *et al.,* 2000; Van den Steen *et al.,* 2002). However, MMPs may also be beneficial to the host by stimulating

protective and adaptive immune responses. Thus, a recent report has revealed that mutant male mice deficient in MMP-8 exhibit an increased tumour susceptibility compared to wild-type mice (Balbin *et al.,* 2003). Histopathological analysis of these *Mmp8*-deficient mice has revealed the presence of abnormalities in the inflammatory response induced by carcinogens. In fact, the lack of this MMP hampers the early stages of inflammation, but once established it is abnormally sustained leading to a more favourable environment for tumour development. The prolonged accumulation of inflammatory cells likely results in chronic inflammation which facilitates genomic instability and promotion of tumour growth (Coussens and Werb 2002). Therefore, and contrary to previous studies performed with mice lacking specific MMPs, loss of MMP-8 enhances rather than reduces tumour susceptibility. A putative mechanism to explain these paradoxical effects of a MMP family member comes from its potential proteolytic processing activity on inflammatory mediators, which could contribute to the host antitumour defense system. We are currently evaluating the possibility that MMP-8 could play a role in the proteolytic inactivation of

TABLE 2

PHENOTYPES OF MICE WITH GENETIC MODIFICATIONS IN THE MMP SYSTEM

Genetically modified mice	Phenotype	Tumour development
Transgenic mice		
Haptoglobin-*Mmp1*	Hyperkeratosis, acanthosis	Increased skin carcinogenesis
WAP-*Mmp3*	Precocious alveolar branching morphogenesis	Increased mammary carcinogenesis
MMTV-*Mmp3*	Mammary epithelial cell apoptosis	Increased mammary carcinogenesis
MMTV-*Mmp7*	Disorganized testis, infertility	Increased mammary carcinogenesis
MMTV-*Mmp14*	Mammary hyperplasia	Increased mammary carcinogenesis
Knock-out mice		
Mmp2$^{-/-}$	Reduced angiogenesis Delayed mammary gland differentiation	Reduced pancreatic carcinogenesis Decreased tumour growth
Mmp3$^{-/-}$	Accelerated mammary gland adipogenesis Delayed incisional wound healing Resistance to contact dermatitis Impaired *ex vivo* herniated disc resorption	
Mmp7$^{-/-}$	Defective innate intestinal immunity Impaired tracheal wound repair Impaired migration of neutrophils Defective prostate involution Impaired *ex vivo* herniated disc resorption	Reduced intestinal adenoma formation
Mmp8$^{-/-}$	Defective inflammatory response	Increased skin carcinogenesis in males
Mmp9$^{-/-}$	Delayed growth plate vascularization Defective endochondral ossification Defective in osteoclast recruitment Resistance to bullous pemphigoid Resistance to aortic aneurysms Prolonged contact dermatitis Abnormal embryonic implantation Protection from cardiac rupture after infarction Diminished neutrophil infiltrate in glomerular nephritis	Reduced skin carcinogenesis Reduced pancreatic carcinogenesis Reduced experimental metastasis Reduced pancreatic carcinogenesis
Mmp11$^{-/-}$	Accelerated neointima formation after vessel injury	Reduced mammary carcinogenesis Decreased tumour cell survival and growth Increased number of metastasis
Mmp12$^{-/-}$	Resistance to cigarette-smoke-induced emphysema	
Mmp14$^{-/-}$	Severe abnormalities in bone and connective tissue Defective angiogenesis Premature death	
Mmp20$^{-/-}$	Amelogenesis imperfecta	

proinflammatory cytokines or chemokines, thereby contributing to the appropriate resolution of inflammatory responses induced by carcinogens.

The role of MMPs in angiogenesis is also dual and complex. The relevance of these enzymes as positive regulators of tumour angiogenesis has been largely demonstrated. Thus, several pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or transforming growth factor-β (TGF-β) are induced or activated by these enzymes, triggering the angiogenic switch during carcinogenesis and facilitating vascular remodelling and neovascularization at distant sites (Belotti *et al.,* 2003; Bergers *et al.,* 2000; Mohan *et al.,* 2000; Sounni *et al.,* 2002; Yu and Stamenkovic 2000). An additional connection between angiogenic factors and MMPs derives from the recent finding that MMP-9 is induced in tumour macrophages and endothelial cells and promotes lung metastasis (Hiratsuka *et al.,* 2002). Furthermore, host-derived MMP-9 contributes to the malignant behaviour of ovarian carcinomas by promoting neovascularization (Huang *et al.,* 2002). However, and contrary to these proangiogenic roles of MMPs, the recent description of mechanisms by which these enzymes negatively regulate angiogenesis has contributed to increase the functional complexity of this proteolytic system in cancer. Thus, a number of MMPs are able to cleave the precursors of angiostatin and endostatin, and generate the active forms of these endogenous inhibitors of angiogenesis (Cornelius *et al.,* 1998; Ferreras *et al.,* 2000). Furthermore, a recent study has correlated the generation of tumstatin by MMP-9-mediated proteolysis of type IV collagen, with the suppression of pathological angiogenesis and tumour growth (Hamano *et al.,* 2003).

Taken together, these findings illustrate the diversity of MMP functions associated with cancer and highlight the importance of MMP protective activities in tumour progression, an aspect that had been largely overlooked in this field. Hence, it is critical to identify the physiological role of each individual MMP and its specific participation in the multiple stages of tumour evolution to better develop effective therapeutic interventions.

Regulation of MMPs

In order to block the undesired activities of MMPs in cancer, it is first necessary to understand the precise mechanisms that regulate MMP expression and activity in both physiological and pathological conditions. Despite the complexity of MMP regulation, three major levels of endogenous control can be recognized: gene transcription, proenzyme activation and inhibition of their enzymatic activity. Collectively, these mechanisms should confine MMP degradative activity to those sites and situations where it is biologically necessary. However, tumour cells have developed multiple strategies to escape these checkpoints controlling the MMP proteolytic activity, acquiring new properties that lead to tumour growth and invasion.

Transcriptional regulation
The absence of a universal mechanism responsible for the observed MMP overexpression in tumours may be a consequence of the multiple cells contributing to the synthesis of these enzymes during cancer evolution. Thus, in addition to their production by epithelial tumour cells, MMP gene expression may be induced in stromal fibroblasts, or in vascular and inflammatory cells that infiltrate tumours (De Wever and Mareel 2003; Nielsen *et al.,* 2001). Accordingly, MMP induction mechanisms appear to be different depending on the characteristics of the diverse cells with ability to produce these enzymes. A wide variety of agents, including cytokines, growth factors and oncogene products cause spatial and temporal variations of MMP expression (Westermarck and Kahari 1999). Nevertheless, TNF-α and IL-1 are regularly implicated in MMP gene induction in different tumours, whereas TGF-β or retinoids usually repress MMP transcription. However, there are several exceptions to this situation, since some family members such as *Mmp11* or *Mmp13* can be induced rather than repressed by these factors in diverse cell types (Guerin *et al.,* 1997; Overall *et al.,* 1989; Uria *et al.,* 1998). It is also possible to find similarities among the signal-transduction pathways mediating induction of different MMPs. Thus, the ERK and the p38 mitogen activated protein kinase pathways are relevant in a number of cases (Pan and Hung 2002; Reunanen *et al.,* 2002; Ruhul Amin *et al.,* 2003; Tanimura *et al.,* 2003).

Structural and functional analysis of the promoter regions from a number of MMP genes has provided a better understanding of the mechanisms that regulate their expression. These studies have revealed the existence of an AP-1 binding site in the promoter of most MMP genes (Pendas *et al.,* 1997). This enhancer element binds homodimers or heterodimers of the Fos and Jun family of oncoproteins, thereby providing an interesting connection between transcription factors related to malignant transformation and MMP expression. Likewise, the PEA3 site which binds the ETS family of oncoproteins, is also present in many MMP gene promoters (Crawford *et al.,* 2001). It has been demonstrated that the ETS and AP-1 binding sites cooperate to enhance transcription, although the presence of other upstream elements such as NF-κB or Cbfa1 binding sites is also necessary to precisely regulate MMP gene expression and tissue specificity (Bond *et al.,* 1998; Jimenez *et al.,* 2001). Finally, it is important to emphasize the presence in several MMP gene promoters, of single nucleotide polymorphisms (SNPs) with ability to influence cancer susceptibility. One of these SNPs identified in the *Mmp1* promoter creates an ETS binding site that enhances transcription of *Mmp1*, and is associated with several cancers (Tower *et al.,* 2003; Wyatt *et al.,* 2002; Zhu *et al.,* 2001). Additional SNPs influencing cancer susceptibility have also been reported in the promoter of other MMPs such as *Mmp2*, *Mmp3* and *Mmp7* (Ghilardi *et al.,* 2002; Ghilardi *et al.,* 2003; Miao *et al.,* 2003; Yu *et al.,* 2002).

Proenzyme activation
MMPs, like most proteolytic enzymes, are synthesized as inactive zymogens. Therefore, the activation of proMMPs represents another step in the regulation of MMP activity. Several agents such as thiol-modifying reagents, mercurial compounds, reactive oxygen radicals, a variety of denaturant agents, as well as conditions of low pH and high temperature, can lead to MMP activation *in vitro* (Nagase 1997). This activation is mainly achieved through the disturbance of the interaction between a cysteine-sulphydryl group in the propeptide domain and the zinc ion bound at the catalytic site. This mechanism, known as the cysteine-switch model, has been supported by structural analysis and represents a general model for maintaining proMMP latency (Morgunova *et al.,* 1999; Van Wart and Birkedal-Hansen 1990). *In vivo*, MMP activation requires the participation of other proteases to remove

the propeptide domain. In most cases, these activating proteases form part of a proteolytic cascade that takes place in the immediate pericellular space (Lijnen 2001). The finding that MT-MMPs are able to activate some proMMPs has provided strong support to this concept (Morrison *et al.,* 2001; Nie and Pei 2003; Sato *et al.,* 1994; Strongin *et al.,* 1995; Zucker *et al.,* 2003). In contrast to the pericellular mechanism of proMMP activation, a set of MMPs, including MT-MMPs, MMP-11, MMP-23 and MMP-28, possesses a furin-like recognition sequence in the propeptide which allows their intracellular activation by furin-like proprotein convertases (Lohi *et al.,* 2001; Pei and Weiss 1995; Yana and Weiss 2000; Zucker *et al.,* 2003). Finally, it is remarkable that alternative MMP activation mechanisms have been recently described. These mechanisms may be based on the formation of an S-nitrosylated derivative with the thiol group of the cysteine switch (Gu *et al.,* 2002), or can be mediated *in vivo* by the MMP binding to a ligand or to a substrate (Bannikov *et al.,* 2002).

Endogenous inhibitors

The activity of MMPs may be also controlled by a series of endogenous inhibitors. Some of them are general protease inhibitors such as α2-macroglobulin, which mainly blocks MMP activity in plasma and tissue fluids, whereas other inhibitors such as TIMPs (tissue inhibitors of metalloproteinases) are more specific. Four TIMPs have been identified in vertebrates (Brew *et al.,* 2000). TIMP-1, TIMP-2 and TIMP-4 are secreted proteins whereas TIMP-3 is anchored in the ECM. All of them share a conserved structure divided into an N- and a C-terminal domain and containing three conserved disulfide bonds (Williamson *et al.,* 1990). Although it had been described that TIMPs reversibly inhibited MMPs in a stoichiometric manner, the mechanism of interaction remained unknown until the 3D structure of the TIMP-1/MMP-3 complex was solved (Gomis-Ruth *et al.,* 1997). This structure has demonstrated that the TIMP-1 N-terminal domain is the main responsible for MMP inhibition through its binding to the catalytic site in a substrate-like manner. The four TIMPs can inhibit the active form of all MMPs tested to date, although TIMP-1 is a poor inhibitor of MMP-19 and of some MT-MMPs (Lee *et al.,* 2003). It is also remarkable the ability of TIMP-3 to block the activity of certain ADAMs (a disintegrin and metalloproteinase) and ADAM-TSs (ADAMs with thrombospondin domains) (Amour *et al.,* 2000; Kashiwagi *et al.,* 2001). The inhibitory activities of TIMPs suggest that the net balance between MMPs and TIMPs is a major determinant of the proteolytic potential of tumours. This concept has been supported by several studies showing that overproduction of TIMPs reduces experimental metastasis (DeClerck and Imren 1994), whereas low levels of these inhibitors correlate with tumorigenesis (Khokha *et al.,* 1989). Moreover, TIMP-2 inhibits endothelial cell proliferation *in vitro* and angiogenesis *in vivo* through a MMP-independent mechanism (Seo *et al.,* 2003). Likewise, TIMP-3 can also block the binding of VEGF to VEGF receptor-2, thereby inhibiting downstream signalling and angiogenesis (Qi *et al.,* 2003). However, several studies have shown that TIMP levels are also increased during tumour progression and may exhibit growth promoting activities on a number of cell types, indicating that their role in cancer progression is much more complex than that derived from MMP inhibitory function (Baker *et al.,* 2002; Jiang *et al.,* 2002).

In addition to the inhibitory action of TIMPs, MMP function may be also blocked by a number of proteins recently described. Some of these novel MMP endogenous inhibitors contain sequences with certain similarity to the N-terminal domain of TIMPs. This is the case of the procollagen C-terminal proteinase enhancer (Mott *et al.,* 2000), the NC1 domain of type IV collagen (Petitclerc *et al.,* 2000), or the tissue factor pathway inhibitor-2 (Herman *et al.,* 2001). Finally, RECK (reversion-inducing cysteine-rich protein with kazal motifs) is a membrane-bound protein with ability to act as a MMP inhibitor (Liu *et al.,* 2003; Oh *et al.,* 2001). Taken together, all these observations reflect the diversity of the MMP endogenous inhibitors and the complexity that can be derived from their activities in physiological and pathological conditions, including cancer.

Strategies for MMP inhibition in cancer therapy

The relationship between MMPs overproduction and tumour progression has prompted the development of a variety of strategies aimed to block the proteolytic activities of these enzymes. However, most clinical trials using MMP inhibitors have yielded disappointing results (Coussens *et al.,* 2002; Overall and Lopez-Otin 2002; Pavlaki and Zucker 2003). The recent recognition of the complex roles that these enzymes play during physiological and pathological conditions may explain the lack of success of the first generation of MMPIs. Accordingly, the increased knowledge on this proteolytic system may lead to the development of new strategies of MMP inhibition, based on targeting any of the three major levels of endogenous regulation of these enzymes: transcription, activation and inhibition (Freije *et al.,* 2003; Overall and Lopez-Otin 2002).

Targeting MMP *gene transcription*

There are three main approaches for targeting MMP gene transcription: preventing the action of extracellular factors, blocking signal-transduction pathways, and targeting those nuclear factors that enhance the expression of the corresponding MMP gene (Westermarck and Kahari 1999). In relation to the first of them, several studies have identified a wide number of factors able to up-regulate the expression of these enzymes in diverse diseases including cancer. However, the diversity of agents that can mediate MMP production as well as the opposite effects of these factors on the expression of different MMP genes, difficults the selection of the appropriate targets. Nevertheless, recent studies have shown that factors such as IFN-α (interferon-α), IFN-β and IFN-γ can be used to inhibit the transcription of several MMPs in diverse human cancer cells (Kuga *et al.,* 2003; Ma *et al.,* 2001; Slaton *et al.,* 2001). Alternatively, different strategies designed for blocking those cytokine-receptor interactions that up-regulate MMP genes have led to interesting results. In fact, several studies have shown that the blockade of TNF-α, IL-1 or epithelial growth factor (EGF) receptors reduce MMP production in arthritis or cancer, validating the usefulness of this approach for blocking MMPs (Lal *et al.,* 2002; Mengshol *et al.,* 2002).

A second general approach to abrogate MMP production consists in targeting the signal-transduction pathways that mediate induction of these enzymes. In this regard, the blockade of specific steps in the MAPK pathway leads to the suppression of MMP gene expression in diverse cancer cells. Thus, selective inhibition of p38 MAPK activity with SB203580 abolishes the expression of MMP-1, MMP-9 and MMP-13 in transformed keratinocytes and squamous cell carcinoma

cells (Johansson *et al.,* 2000; Simon *et al.,* 1998). Likewise, the specific blockade of the ERK pathway has led to MMP down-regulation in tumour cells (Pan and Hung 2002; Tanimura *et al.,* 2003). Other compounds such as halofuginone, manumycin A, and malolactomycin D also block MMP gene expression through the interference with the TGF-β or Ras signalling pathways (Futamura *et al.,* 2001; McGaha *et al.,* 2002; Zhang *et al.,* 2002).

A third option to block MMP up-regulation in human tumours is to target the nuclear factors directly responsible for MMP transcription. Strategies designed to block general factors such as AP-1 and NF-κB by using glucocorticoids (Karin and Chang 2001) or certain natural products (Aggarwal *et al.,* 2003; Sato *et al.,* 2002; Shishodia *et al.,* 2003; Takada and Aggarwal 2003; Woo *et al.,* 2003a; Woo *et al.,* 2003b), have demonstrated their ability to suppress the production of many MMPs in different cancer types. However, these strategies affect the expression of multiple genes and may have several side effects that could be avoided by targeting more specific factors such as Cbfa1, which selectively modulates the expression of certain MMPs (Jimenez *et al.,* 2001; Yang *et al.,* 2001a). In addition, restoration of the activity of several tumour suppressors such as p53, PTEN, and TEL which are lost in multiple cancers, decreases MMP expression (Fenrick *et al.,* 2000; Koul *et al.,* 2001; Sun *et al.,* 2000). Finally, inhibition of MMP synthesis by antisense-gene transfer constructs (Kondraganti *et al.,* 2000; London *et al.,* 2003), ribozymes (Hua and Muschel 1996), and RNA interference-based approaches (Sanceau *et al.,* 2003; Ueda *et al.,* 2003) represent gene-selective strategies of potential interest for cancer therapy.

Blocking proMMP activation

MMP gene expression is followed by the participation of multistep proteolytic cascades that finally render the active enzyme. This fact implies that there are several new possibilities of MMP inhibition based on targeting proMMP activation. Several strategies in this regard have been designed to block MT1-MMP, because of its ability to activate proMMPs and also because of its central role in regulating tumour growth (Hotary *et al.,* 2003; Seiki 2003; Sounni *et al.,* 2003). Hence, anti-MT1-MMP monoclonal antibodies, that inhibit its proteolytic activity and impair endothelial cell migration and invasion of collagen and fibrin gels, could be used in future clinical trials (Galvez *et al.,* 2001). MT1-MMP dependent activation of proMMP can also be blocked by natural products such as green-tea catechins (Annabi *et al.,* 2002). Furthermore, the complexity of the enzymatic cascade of MMP activation provides new possibilities to target tumour MMPs by blocking the upstream activators of proMT-MMPs. In this regard, a selective furin inhibitor such as α1-PDX prevents MT1-MMP activation and proMMP-2 processing, with the subsequent attenuation of tumourigenicity and invasiveness of human cancer cells (Bassi *et al.,* 2001). Alternative strategies to block MMP activation are based on the use of thrombospondin-1, which binds to proMMP-2 and proMMP-9 and directly blocks their activation (Bein and Simons 2000; Rodriguez-Manzaneque *et al.,* 2001), or thrombospondin-2, which forms a complex with proMMP-2 and promotes its endocytosis (Yang *et al.,* 2001b). Likewise, endostatin (Kim *et al.,* 2000; Nyberg *et al.,* 2003) and proteoglycans such as testican-3 and N-Tes (Nakada *et al.,* 2001) can suppress proMMP-2 activation mediated by MT1-MMP. Finally, protease inhibitors used in human immunodeficiency virus (HIV) therapy are also able to block proMMP-2 activation, thereby contributing to the

regression of highly aggressive tumours, such as Kaposi's sarcoma, occurring in HIV patients (Sgadari *et al.,* 2002).

Inhibition of active MMPs
Therapeutic potential of TIMPs

The potential application of TIMPs to block the MMP activity in cancer was initially supported by several studies demonstrating their ability to inhibit tumour growth in transgenic mouse models (Kruger *et al.,* 1997; Martin *et al.,* 1999). However, the possibility of using TIMPs in cancer therapy has technical difficulties, as it happens with other macromolecules (Overall and Lopez-Otin 2002). In addition, recent studies have revealed a series of paradoxical effects of these proteins derived from their ability to perform functions distinct of MMP inhibition. Thus, TIMP-4 up-regulates the anti-apoptotic protein Bcl-X$_L$, thereby stimulating mammary tumourigenesis (Jiang *et al.,* 2001), whereas TIMP-2 shows cell-growth promoting activity (Baker *et al.,* 2002; Jiang *et al.,* 2002). Furthermore, TIMPs are broad-spectrum inhibitors of MMPs and may block the activity of those MMPs that are not necessarily overexpressed in a particular tumour or play protective roles against cancer (Balbin *et al.,* 2003). These observations highlight the need for developing synthetic MMPIs that selectively target specific MMPs.

Synthetic inhibitors and clinical trials

Although the regulatory mechanisms that control MMP production and activity offer new possibilities for therapeutic intervention, most clinical trials for targeting MMPs have been designed to directly block the proteolytic activity of these enzymes. The first series of synthetic inhibitors were pseudopeptides mimicking the cleavage sites of MMP substrates. They contained a zinc-binding hydroxamate moiety which inhibited MMP activity by specifically interacting with the Zn^{2+} in the catalytic site. Thus, Batimastat (BB-94), a broad-spectrum hydroxamate-based inhibitor, became the first MMPI to be tested in humans (Wojtowicz-Praga *et al.,* 1996). However, clinical trials with intraperitoneally administered Batimastat did not shown any significant responses, and it was replaced by Marimastat (BB-2516), another peptido-mimetic MMPI but orally available. Marimastat inhibits the activity of many MMPs including MMP-1, -2, -3, -7, -9, -12, and -13. The number of distinct enzymes that this MMPI can target could explain the musculoskeletal pain detected in patients after a sustained treatment with Marimastat (Nemunaitis *et al.,* 1998). Despite this limitation, Marimastat is as effective as conventional therapy (gemcitabine) in treatment of pancreatic carcinoma patients (Bramhall *et al.,* 2001). Furthermore, this inhibitor in combination with temozolomide, improves survival in patients with glioblastoma multiforme (Groves *et al.,* 2002). Lastly, Marimastat can increase survival and time to disease progression in patients with advanced gastric cancer (Bramhall *et al.,* 2002).

Recently, new series of non-peptido mimetics MMPIs with increased specificity and oral bioavailability and based on the 3D structure of MMP zinc-binding sites have been synthesized. Among them, BMS-275291, has special interest due to lack of musculoskeletal side effects and it is currently being evaluated in advanced lung cancer, prostate cancer and AIDS-related Kaposi's sarcoma (Lockhart *et al.,* 2003). In addition, non-peptidic substances with inhibitory properties against MMPs, including tetracycline derivatives and bisphosphonates are being tested in clinical trials (Cianfrocca *et al.,* 2002; Falardeau *et al.,* 2001; Lacerna and Hohneker 2003). In summary, despite initial problems with MMPIs, the stimulating results obtained with marimastat

are a proof of principle on the clinical value of these compounds for future cancer treatment.

Novel approaches for MMP inhibition

Taking advantage of the frequent overproduction of MMPs in malignant tumours, novel strategies that exploit the catalytic functions of these enzymes have been recently described for cancer therapy. Some of these approaches involve the generation of protease-activatable retroviral vectors which contain engineered MMP-cleavable linkers (Peng *et al.,* 1999; Schneider *et al.,* 2003). Other strategies employ macromolecular carriers that are linked to anticancer drugs released from the carrier by the proteolytic activities of MMPs present in the tumour environment (Mansour *et al.,* 2003). Likewise, Hayashi *et al.,* have designed carriers linked to bioactive molecules that stimulate the antitumour immune response when are liberated by tumour MMPs (Hayashi *et al,* 2002). Finally, a mutated cytotoxin has been engineered by replacing the furin protease cleavage site that is involved in lethal-factor activation with sequences that are selectively cleaved by MMPs (Liu *et al.,* 2000). The optimization of linker peptides design offers a variety of possibilities for cancer therapy based on expression patterns of MMPs in malignant tumours. Another interesting alternative to synthetic MMPIs is the use of gene therapy approaches aimed at delivering TIMPs at tumour sites (Baker *et al.,* 2002; Zacchigna *et al.,* 2004). However, in addition to the current limitations of gene therapy which include low transfer efficiency and poor specificity of response, the paradoxical effects of TIMPs in cancer may hamper the future clinical application of this approach. On the other hand, it should also be possible to develop innovative strategies for MMP targeting in cancer based on the use of 'exosite blockers'. Protease exosites are substrate-binding sites that lie outside the active-site cleft of the enzyme but are crucial for its proteolytic efficiency (Overall 2002). In the case of MMPs, it should be feasible to design exosite inhibitors that target substrate-specific binding sites located in some of the ancillary domains of these proteases (Fig. 1), thereby reducing the binding and cleavage of specific substrates by the corresponding MMP. Likewise, recent experiments have shown that the C-terminal hemopexin domain of MT1-MMP binds native collagen and blocks the collagenolytic activity of both MMP-2 and MT1-MMP (Tam *et al.,* 2002). These findings have opened the possibility of designing substrate-targeted inhibitors that bind the substrate, competing for protease binding at exosites or masking the cleavable peptide bonds. These examples of noncatalytic targeting of MMPs may be part of alternative and innovative strategies aimed at blocking the unwanted activity of these enzymes during tumour progression.

Conclusions and perspectives

The overproduction of MMPs in cancer has long been correlated with tumour progression and metastasis. Therefore, it is not surprising that over the last years MMPs have been the focus of multiple anticancer trials. The lack of success of most of these clinical trials which were based on using broad-spectrum MMPIs in patients with advanced cancer, has made necessary a reformulation of the role of this proteolytic system in cancer. A series of recent works mainly performed with mouse models of gain and loss of MMP function have provided strong support to the idea that these enzymes play essential roles in early stages of cancer (Fig. 2). These studies have also revealed that certain MMPs can have dual
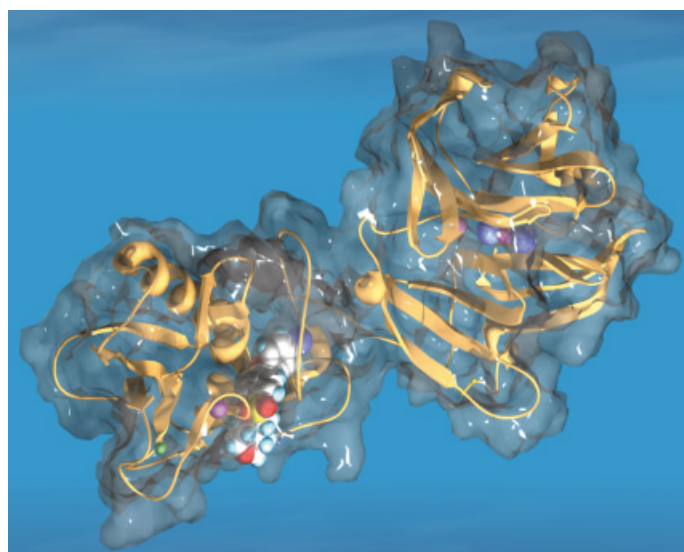
Fig. 3. Structural model of human collagenase-3 bound to a selective inhibitor. *The model was created combining the structural data from the catalytic domain (pdb code 830C) and the hemopexin domain (pdb code 1PEX). Zn^{2+} ions are shown in green, Ca^{2+} ions in pink, and Cl^- ions in blue.*

effects on cancer development (Andarawewa *et al.,* 2003) or even favour the host instead of the tumour (Balbin *et al.,* 2003; Hamano *et al.,* 2003; Pozzi *et al.,* 2002). Therefore, broad-spectrum MMPIs may interfere with the natural host defence mechanism against tumours involving bioactive molecule processing by MMPs (Balbin *et al.,* 2003). Moreover, these MMPIs also target proteases such as the ADAMTSs, which have the ability to slow tumour growth through their antiangiogenic activity (Vazquez *et al.,* 1999). Taken together, these findings provide explanations to previous failures of clinical trials with MMPIs (Coussens *et al.,* 2002; Overall and Lopez-Otin 2002; Pavlaki and Zucker 2003), and emphasise the importance of defining the cancer degradome: the complete set of proteases produced by a specific tumour at a certain stage of development (Lopez-Otin and Overall 2002). This concept could be helpful to precisely identify the set of proteases that must be targeted in each specific situation, especially in light of the above mentioned findings demonstrating the occurrence of "protective" enzymes preventing tumour progression (Balbin *et al.,* 2003).

The identification of the specific proteases that must be targeted in cancer should also be correlated with the design of MMPIs that selectively reduce the binding and cleavage of certain substrates by the protease, while not interfering with the cleavage of others. For this purpose, it is essential to increase the number of 3D structures available for these enzymes (Fig. 3), as well as to identify the *in vivo* substrates that MMPs can target alone or in cooperation with other proteolytic systems and whose hydrolysis may strongly influence the behaviour of tumour cells (Table 2). In addition, a better understanding of the regulatory mechanisms that control MMP transcription, activation and inhibition may offer innovative strategies for targeting MMPs in cancer. These basic studies together with clinical improvements, such as introduction of imaging technologies for *in vivo* detection of MMPs, identification of surrogate markers of MMP inhibition, and design of appropriate combinations of MMPIs with cytotoxic drugs, may finally lead to effective MMPI-based therapies for cancer.

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