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EFFECT OF BISPHOSPHONATES AND SMALL CYCLIC PEPTIDES ON MATRIX METALLOPROTEINASES AND HUMAN CANCER CELLS

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Academic dissertation

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Abbreviations

AEC	3-amino-9-ethylcarbazole
AGRE	AG-rich element
α2M	α2-macroglobulin
AP	alkaline phosphatase
AP-1	activator protein 1
APMA	p-aminophenylmercuric acetate
BCIP	5-bromo-chloro-3-indolyl-phosphate
bFGF	basic fibroblast growth factor
BM	basement membrane
BSA	bovine serum albumin
CBFA1	core-binding factor A 1
CIZ	zinc-finger protein
CMT	chemically modified tetracycline
CTIBL	cancer-treatment-induced bone loss
CTT1	CTTHWGFTLC
CTT2	GRENYHGCTTHWGFTLC
3D	three dimensional
DAPI	2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EMMPRIN	extracellular matrix metalloproteinase inducer
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
GPI	glycosylphosphatidylinositol
HNC	head and neck cancer
HSC-3	human squamous cell carcinoma cell line
HUVEC	human umbilical vein endothelial cells
ICC	immunocytochemistry
IFN	interferon
IGF-BP	insulin-like growth factor binding protein
IL	interleukin
kDa	kilodalton
KGF	keratinocyte growth factor
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
MNC	mononuclear cell
mRNA	messenger ribonucleic acid

MT	membrane-type
NGF	nervous growth factor
NF-κB	nuclear factor kappa B
NSAID	non-steroidal anti-inflammatory drug
NTB	nitro blue tetrazolium
OSE2	osteoblast-specific element
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PEA3	polyoma virus enhancer A binding protein 3
PTEN	phosphatase and tensin homologue
RPA	ribonuclease protection assay
SAPK/JNK	stress activated proteinase kinase/Jun N-terminal kinase
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT	signal transducers and activators of transcription
TCF4	T-cell factor 4
TGF-α, β	transforming growth factor alfa, beta
TEL	translocation-ETS-leukaemia
TIE	TGF-β inhibitory element
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumour necrosis factor α
uPA	urokinase-type plasminogen activator
VEGF	vasculature endothelial growth factor

Abstract

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases that are associated with the tumourigenic process. MMPs can degrade almost all extracellular matrix and basement membrane components, promoting tumour invasion and metastasis. They can also regulate and modify host defence and immune mechanisms as well as normal cell function, and therefore excessive blockage or inhibition of all MMPs may not lead to a positive therapeutic outcome. Most clinical trials with MMP inhibitors (MMPIs) have yielded disappointing results, perhaps due to inappropriate study design or tumour staging, or to the lack of selectivity. Nonspecific or broad-spectrum MMPIs seemingly affect several members of the MMP family causing side effects during antitumour therapy because of disruption of numerous physiological or defensive processes.

Bisphosphonates were shown to be broad-spectrum inhibitors of MMPs inhibiting MMP-1, -2, -3, -8, -9, -12, -13 and -20 and this inhibition was found to involve cation chelation. Bisphosphonates were further shown to exert anti-metastatic, anti-invasive and cell adhesion-promoting properties, which may eventually prevent metastases not only in hard tissues but in soft tissues as well in a dose-dependent manner. Clodronate, at therapeutically attainable concentrations, dose-dependently inhibited directly the activity of catalytic domain of human recombinant MT1-MMP, reduced the activation of proMMP-2 and downregulated the expression of MT1-MMP mRNA and protein production in MG-63 human osteosarcoma cells.

Two novel decapeptides, CTT1 and CTT2, were generated and characterized to target selectively and specifically gelatinases (MMP-2 and -9), inhibiting their gelatinase activities in several human carcinoma cell lines and in endothelial cells both *in vitro* and *in vivo*. Furthermore, CTT1 and CTT2 reduced tumour growth and increased survival in a mouse model. It is therefore to be hoped that these novel gelatinase-specific MMPIs will in the future open up a new era in the medical treatment of cancer. The pivotal role of MMPs in pathological conditions demonstrates that the MMPIs are still attractive for drug development.

1. Introduction

Matrix metalloproteinases (MMPs) form an enzyme family capable of degrading almost all constituents of the extracellular matrix (ECM) and the basement membrane (BM). 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 and BM components (Egeblad and Werb 2002, Freije *et al.* 2003, Hojilla *et al.* 2003). MMPs can also regulate multiple cellular functions including cell growth, apoptosis, angiogenesis, invasion, metastasis and immune response by cleaving growth factor-precursors, cell adhesion molecules and other bioactive proteins including MMPs themselves. The MMP family includes collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs) and other MMPs. Pathologically excessive expression of MMPs has been implicated in the processes of tumour growth, invasion and metastasis (Egeblad and Werb 2002).

Over the past 20 years, the pharmaceutical industry has made extensive efforts to develop synthetic matrix metalloproteinase inhibitors (MMPIs) for the treatment of cancer and other tissue-destructive diseases (Baker *et al.* 2002). Most of the early anti-MMP drugs were designed as peptides mimicking of the collagen amino-acid sequence near the collagenase cleavage site, probably because at the time collagen degradation was viewed and regarded as a pivotal and key rate-limiting point during tumour growth and progression (Brown 2000). It is now known that the diversity of MMP functions associated with cancer highlight the importance of protective activities of MMPs in tumour progression, an aspect that had seemingly been overlooked. Hence, it seems important to try to identify the physiological role of each individual MMP and its specific participation in the multiple and complex stages of tumour evolution in order to develop effective and hopefully selective therapeutic interventions (Overall and Lopez-Otin 2002).

In this work, I studied the role of certain MMPs and MMPIs in tumour progression, but it should be kept in mind that these MMPs are not the only proteolytic contributors in these processes, and interactions between members of other classes of proteolytic enzymes, such as matrix destructive serine proteinases eventually provide additional complexity and regulation of proteolytic cascades associated with malignancies (Sorsa *et al.* 1997, Moilanen *et al.* 2003).

1.1. Structure of extracellular matrix (ECM)

ECM forms complex, highly organized structures that provide support for both tissues and individual cells regulating their physical properties. ECM also regulates cell behaviour influencing their adhesion, migration, proliferation, shape, development and metabolic functions. The ECM is not a static structure, but rather constantly produced, remodelled and processed.

The ECM contains collagens, non-collagenous glycoproteins and proteoglycans. These structural macromolecules are largely secreted by fibroblasts. In more specialized tissues, such as bone and cartilage, the extracellular matrix is secreted by mesenchymal cells, such as chondroblasts in cartilage and osteoblasts in bone. The long collagen fibres strengthen and organize the matrix, while the polysaccharides of the proteoglycans form an aqueous phase, which permits the diffusion of nutrients, metabolites and hormones between tissue compartments. Elastin, fibronectin and laminin are among the major components of the ECM. Fibronectin is widely distributed in connective tissues, whereas laminin is found exclusively in the BM. Fibronectin is a large glycoprotein and has multiple domains, each with specific binding sites for other matrix macromolecules and for receptors on the surface of cells. It therefore contributes to both organizing the matrix and helping cells attach to it. Fibronectin is important not only for cell adhesion to the matrix but also for guiding cell migrations in vertebrate embryos (Alberts *et al.* 2002). Vitronectin, thrombospondin, tenascin, and SPARC (secreted protein, acidic and rich in cysteine) are other ECM glycoproteins.

The major proteins present in the ECM are collagens. To date, 30 different collagen α-chains differing in the primary sequence have been characterized, that are composed of three α-chains and contain at least one triple-helical domain of repeating glycines (Gly-X-Y motif). Insoluble collagen fibrils make up about 30% of the proteins in the human body (Aumailley and Gayraud 1998, Myllyharju and Kivirikko 2001). Fibrillar collagens (types I, II, III, V and XI) form fibrils and influence cellular functions through interactions with integrins and are the main collagen types found in connective tissue. Type I collagen is the most abundant protein in the human body and it is the major collagen in skin, bone, tendon and ligaments (Prockop and Kivirikko 1995).

1.1.1. Basement membranes

BMs are a 50- to 100-nm thick layer of highly specialized ECMs. They separate epithelial or endothelial cells from the adjacent connective tissue or surround groups of cells in e.g. fat, nerve and muscle tissue. BMs have highly specialized mechanical and biological functions. They provide physical support for tissues, serve as a physiological barrier for cells of different origin, regulate cell polarity and are involved in cell differentiation and migration as well as tissue repair and remodeling. BMs also act as reservoirs of plasma proteins, enzymes and growth factors (Yurchenco and O'Rear, 1994). Major BM components include type IV collagen, laminin, nidogen (entactin) and proteoglycans. Minor components include agrin, SPARC, fibulins, type XV collagen and type XVIII collagen (Erickson and Couchman 2000, Ghohestani *et al.* 2001). Type IV collagen and laminin both exist as multiple isoforms, each forming a huge irregular network by self- assembly. These networks are connected by nidogen, which also binds to several other components (proteoglycans and fibulins). BMs contain at least two kinds of proteoglycans, heparin sulfate proteoglycans and chondroitinsulfate proteoglycans. BMs are connected to cells by several receptors of the integrin family, which bind preferentially to laminins and collagen IV, and via some

lectin-type interactions (Timpl and Brown 1996). Laminins are a family of at least 15 heterotrimeric glycoproteins composed of five α, three β and three γ subunits. They are involved in many biological functions, such as regulation of tissue morphogenesis, cell differentiation, adhesion and migration (Colognato and Yurchenco 2000).

1.2. The functional and structural properties of matrix metalloproteinases (MMPs)

Proteolytic enzymes are either exopeptidases, cleaving a substrate molecule's terminal peptide bond, or endopeptidases, cleaving an internal peptide bond of the substrate. Endopeptidases are divided into serine, cysteine, aspartic and metalloproteinases based on their catalytic properties and inhibitor sensitivities (Stöcker *et al.* 1995). The urokinase type plasminogen activator (uPA), mentioned in this study, belongs to serine proteinases containing a serine residue in their catalytic site. UPA converts the plasma protein plasminogen into active plasmin, which has wide substrate specificity and is able to activate several latent proMMPs (Silverman *et al.* 2001).

To date, 24 different vertebrate MMPs have been identified of which 23 are found in humans (Puente *et al.* 2003). MMPs are Ca^{2+} - and Zn^{2+} -dependent endopeptidases, that can collectively cleave most ECM and BM macromolecules (Table 1). MMP numbering is usually determined by the order of the discovery, MMP-1 being the first. The MMPs share high protein sequence homology and have defined domain structures and thus, according to their structural properties, the MMPs are classified as either secreted MMPs or membrane-anchored MMPs, which are further divided into eight discrete subgroups: five are secreted and three are membrane-type MMPs (MT-MMPs) (Fig. 1). MMPs consist of a single polypeptide, varying between 20-100 kDa in size. All MMPs are synthesized with a prodomain containing a leader sequence, which targets the protein for secretion. They are secreted as latent proforms, with a few exceptions of furin-processed family members MMP-11, MMP-23 and MMP-28. The propeptide contains a conserved sequence PRCGXPD, in which the cysteine forms a covalent bond (cysteine switch) with the catalytic zinc (Zn^{2+}) to maintain the latency of proMMPs. The catalytic domain contains the highly conserved zinc binding site HEBXHXBGBXHS motif, where H is histidine, E is glutamic acid, B is bulky hydrophobic amino acid, G is glycine, X is variable amino acid and S is serine in which zinc is coordinated by three histidines. The serine can also be replaced by threonine in certain MMPs, i.e. MMP-11 (Stöcker *et al.* 1995). The proline-rich hinge region links the catalytic domain to the hemopexin domain. The role of the hinge region in MMPs is unclear, but it has been reported that mutations in the MMP-8 hinge region affect autoproteolysis and substrate specificity (Knäuper *et al.* 1997). The hemopexin domain is absent in MMP-7 (matrilysin) and MMP-26 (matrilysin-2, endometase). MMP-2 and MMP-9 (gelatinases A and B, respectively) contain three repeats of the fibronectin-type II domain inserted in the catalytic domain. MT1-, MT2-, MT3- and MT5-MMP contain a transmembrane domain and MT4- and MT6-MMPs contain a

glycosylphosphatidylinositol (GPI) anchor in the C-terminus of the molecule, which attach these MMPs to the cell surface. MT-MMPs, MMP-11, MMP-23 and MMP-28 contain a furin cleavage site (RXKR) between the propeptide and catalytic domain, making these proenzymes susceptible to activation by intracellular furin-convertases. MMP-23 contains an N-terminal signal anchor, which anchors proMMP-23 to the Golgi complex and has a different C-terminal domain instead of hemopexin-like domain (Fig. 1) (Sternlicht and Werb 2001).

The secreted MMPs can also localized onto the cell surface by binding to integrins or to CD44, or through interactions with cell-surface-associated heparan sulphate proteoglycans, collagen type IV or the extracellular matrix metalloproteinase inducer (EMMPRIN) (Brooks *et al.* 1996, Yu and Stamenkovic 1999, Sternlicht and Werb 2001).

1.2.1. Collagenases

The three mammalian collagenases are MMP-1 (collagenase-1), MMP-8 (collagenase-2) and MMP-13 (collagenase-3), while collagenase-18 has been characterized from the frog. Collagenases cleave the native fibrillar collagens I, II, and III at a specific site three-fourths from the N-terminus. The cleavage takes place in a specific site between the glysine-isoleusine (Gly-Ile) of the α1 chain and the glysine-leusine (Gly-Leu) residues of the α2 chain forming the characteristic αA (75%)- and αB (25%)- triple helical cleavage fragments that in body temperature denaturate into randomly coiled gelatin, being further degraded by other gelatinolytic MMPs and other proteinases (Birkedal-Hansen *et al.* 1993, Sternlicht and Werb 2001). The collagenases differ in their substrate specificities and functional roles (Sorsa *et al.* 2004, Owen *et al.* 2004). MMP-1 preferably degrades collagen III, MMP-8 prefers type I collagen, and MMP-13 prefers collagen II (Birkedal-Hansen *et al.* 1993, Knäuper *et al.* 1996a). Collagenases can also digest a number of other ECM and non-ECM molecules (Table 1). Furthermore, native triple helical type I and II collagens can be directly degraded by human tumour-associated trypsin-2 (Moilanen *et al.* 2003, Stenman *et al.* 2004)

MMP-1 was the first MMP found from the metamorphosing tadpole (Gross and Lapiere 1962). MMP-1 is expressed by fibroblasts, endothelial cells, macrophages, hepatocytes, chondrocytes, osteoblasts, tumour cells and migrating epidermal keratinocytes and its expression can be induced in certain inflammatory diseases and cancers (Birkedal-Hansen *et al.* 1993, Meikle *et al.* 1992, Giambernardi *et al.* 1998).

MMP-8 was first cloned from messenger RNA (mRNA) extracted from the peripheral leucocytes of a patient with chronic granulocytic leukaemia (Hasty *et al.* 1987, 1990). MMP-8 is synthesized in polymorphonuclear leukocytes during their maturation in bone marrow and stored in specific intracellular granules, out of which it is secreted to the cell environment in response to external triggering stimuli (Hasty *et al.* 1987, 1990, Ding *et al.* 1997, 1996). MMP-8 can also be detected in mucosal fibroblasts, SCC cells of the tongue, chondrocytes, odontoblasts, monocyte/macrophages, melanoma cells, leukaemia cells, malignant plasma cells and human endothelial cells (Moilanen *et al.* 2003,

Cole *et al.* 1996, Palosaari *et al.* 2000, Giambernardi *et al.* 1998, Kim *et al.* 2002, Wahlgren *et al.* 2001, Hanemaaijer *et al.* 1997, Kiili *et al.* 2002). MMP-8 is expressed *in vivo* by bronchial epithelial cells and macrophages involved in bronchiectasis, oral SCCs, chondrocytes in rheumatoid arthritic and osteoarthritic lesions, rheumatoid synovial fibroblasts, in human gingival sulcular epithelial cells, in cells of human atheroma, in plasma cells associated with oral keratocysts and by cells in proliferating and migratory wound epithelia, in dermal fibroblasts and inflammatory cells (Prikk *et al.* 2001, Moilanen *et al.* 2003, Chubinskaya *et al.* 1999, Hanemaaijer *et al.* 1997, Tervahartiala *et al.* 2000, Herman *et al.* 2001, Wahlgren *et al.* 2001, Pirilä *et al.* 2001). ProMMP-8 can be activated by reactive oxygen species (Saari *et al.* 1990), human trypsin-2 (Moilanen *et al.* 2003), MT1-MMP (Holopainen *et al.* 2003), MMP-3 (Knäuper *et al.* 1993) and bacterial proteases (Sorsa *et al.* 1992).

MMP-13 was originally cloned from human breast tumour cDNA library (Freije *et al.* 1994). It has the widest substrate selection among the interstitial collagenases and, in addition to collagens, it is able to cleave various BM components. MMP-13 cleaves type II collagen more efficiently than type I and III, and among interstitial collagenases, it is most effective in cleaving gelatin (Mitchell *et al.* 1996, Lindy *et al.* 1997). The physiological expression of MMP-13 seems to be limited only to developing bone (Ståhle-Bäckdahl *et al.* 1997), wound healing and teeth (Ravanti *et al.* 1999, Pirilä *et al.* 2001, Sulkala *et al.* 2004). It is widely expressed in pathological conditions including rheumatoid arthritis, osteoarthritis, periodontitis, chronic ulcerations, SCC, hypertrophic chondrocytes, osteoblasts as well as plasma cells and many carcinoma and melanoma cells (Lindy *et al.* 1997, Vaalamo *et al.* 1997, Uitto *et al.* 1998, Johansson *et al.* 2000, Tervahartiala *et al.* 2000, Wahlgren *et al.* 2001, Mitchell *et al.* 1996, Johansson *et al.* 1997a, b, Giambernardi *et al.* 1998, Uria *et al.* 1998, Bachmeier *et al.* 2000, Hofmann *et al.* 2000, Kiili *et al.* 2002). MMP-13 is predicted to have an important role in tumour invasion and metastasis due to its wide substrate-specificity together with catalytic efficiency and its upregulated expression in cancer cells (Kähäri and Saarialho-Kere 1999).

1.2.2. Gelatinases

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) (also called type IV collagenases) are highly efficient in cleaving gelatin along with type IV collagen and several other substrates (Table 1) (Birkedal-Hansen 1993). Gelatinases have been intensively studied in cancer and other diseases.

MMP-2 was the first identified type IV collagenase purified from a malignant murine PMT sarcoma cell line and found to be a potent BM type IV collagen degrading enzyme (Salo *et al.* 1983). MMP-2 is typically expressed constitutively by various cell types, i.e. dermal fibroblasts, keratinocytes and endothelial cells (Birkedal-Hansen 1993), and its expression is associated with many different cell types and cancers, such as melanoma and fibrosarcoma (Huhtala *et al.* 1991) and can be observed in numerous cultured carcinoma cells (Giambernardi *et al.* 1998). Although MMP-2 null mice do not have any apparent abnormalities, mutations in MMP-2 cause bone resorption and arthritis, suggesting an important role for

MMP-2 in human osteogenesis (Martignetti *et al.* 2001). MMP-2 expression has been shown to relate to lymph node metastasis in oral SCC (Kusukawa *et al.* 1993). In carcinomas, the MMP-2 is often derived from the surrounding stromal cells and not from tumour cells (Pyke *et al.* 1992, Soini *et al.* 1993). MMP-2 is involved in many processes that require ECM remodelling, and its overexpression is closely connected to cell migration and to the invasive and metastatic potential of malignant tumours (Visse and Nagase 2003, Giannelli *et al.* 1997).

MMP-9 was identified as a gelatine-binding protein synthesized by human macrophages (Vartio *et al.* 1982), and the MMP-9 gene was cloned from the HT1080 fibrosarcoma cell line (Huhtala *et al.* 1991). The substrate specificity of MMP-9 is very, but not completely, similar to MMP-2, although it does not degrade type I-III collagens as widely as MMP-2 (Table I). It is synthesized during late stages of PMN neutrophil development, stored within the tertiary granules and releases upon stimulus. In other cell types MMP-9 expression requires transcriptional activity (van den Steen *et al.* 2002). MMP-9 is expressed by keratinocytes, T-lymphocytes, alveolar macrophages, monocytes and plasma cells (Salo *et al.* 1991, 1994, van den Steen *et al.* 2002). MMP-9 plays an essential role in reproduction, growth and development, and its overexpression is also connected to the inflammatory reaction, such as lung and periodontal diseases (Westerlund *et al.* 1996, van den Steen *et al.* 2002, Prikk *et al.* 2001, Westerlund *et al.* 1996). MMP-9 plays an important role in tumour cells, invasion and their metastatic potential (Stetler-Stevenson 1990, Giambernardi *et al.* 1998, Thomas *et al.* 2001, Vihinen and Kähäri 2002).

MMP-2 and MMP-9 are in many respects highly similar enzymes, but significant differences exist in the regulation of expression, glycosylation, proenzyme activation and substrate specificities. Despite their largely overlapping functions, MMP-2 and MMP-9 may even have opposing biological activity, as illustrated by the finding that MMP-2 promotes platelet aggregation, while MMP-9 inhibits the same process (Fernandez-Patron *et al.* 1999).

1.2.3. Stromelysins and stromelysin-like MMPs

MMP-3 (stromelysin-1) and MMP-10 (stromelysin-2) have similar substrate specificities, but MMP-3 exerts a higher proteolytic efficiency than MMP-10 (Visse and Nagase 2003) (Table I). Stromelysin-3 (MMP-11) and human macrophage metalloelastase (MMP-12) are often included in a subgroup of stromelysin-like MMPs. MMP-3 and -10 are expressed by keratinocytes *in vivo* and fibroblasts in culture (Giambernardi *et al.* 1998, Johansson *et al.* 2000, Kähäri and Saarialho-Kere 1999). MMP-11 is expressed in breast cancer, and it can degrade serine proteinase inhibitors (serpins) and α1-proteinase inhibitor, but not ECM components (Pei *et al.* 1994).

Metalloelastase (MMP-12) was initially found in alveolar macrophages of cigarette smokers (Shapiro *et al.* 1993). The expression of MMP-12 is also found *in vivo* in macrophages in granulomatous skin diseases, solar elastosis, in intestinal ulcerations and inflammation (Vaalamo *et al.* 1998, Salmela *et al.* 2001, Chung *et al.* 2002). MMP-12 is expressed in carcinoma cells of vulva and in skin cancers (Kerkelä *et al.* 2000). MMP-12 is able to cleave plasminogen into

angiostatin, thus preventing tumour growth by inhibiting angiogenesis (Dong *et al.* 1997, Cornelius *et al.* 1998).

1.2.4. Matrilysins

MMP-7 (matrilysin-1) and MMP-26 (endometase/matrilysin-2) both lack the hinge region and hemopexin domains, which restricts their substrate specificity (Table I). MMP-7 is produced by sweat and salivary glands, airway ciliated cells and the ductal or glandular epithelium of breast, liver, pancreas and urogenital tissues. MMP-7 is also expressed by malignant epithelial cells in tumours of the gastrointestinal tract, prostate and breast (Wilson and Matrisian 1996). Besides ECM components, MMP-7 processes cell surface molecules, pro-α-defensin, Fas-ligand, pro-tumour necrosis factor (TNF)-α and E-cadherin (Visse and Nagase 2003).

MMP-26 was originally isolated from human endometrial tumour library. It is expressed in human placenta and uterus as well as in various tumour cells (Uria and Lopez-Otin 2000). MMP-26 also digests a number of ECM components and can activate proMMP-9 (Visse and Nagase 2003).

1.2.5. Membrane-type MMPs

The membrane-type MMPs contain six members, of which MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16) and MT5-MMP (MMP-24) are bound to the cell membrane with a transmembrane domain and contain a C-terminal cytosolic domain. MT4-MMP (MMP-17) and MT6-MMP (MMP-25) are bound to the cell surface with a C-terminal hydrophobic extension that acts as a GPI anchor (Sternlicht and Werb 2001). MT-MMPs have a furin-sensitive RXKR motif between the propeptide and the catalytic domain, which is cleaved in trans-Golgi network leading to activation of MT-MMPs (Sato *et al.* 1996). MT1-MMP was found on the surface of invasive lung cancer cells with the ability to activate proMMP-2 (Sato *et al.* 1994). MT-MMPs modulate cell-matrix interactions in cell invasion suggesting a marked role in tumour spread. MT1-MMP is expressed in various human cancers, including colon, head and neck carcinomas, liver metastases and hepatocellular carcinomas (Harada *et al.* 1998, Theret *et al.* 1998, Sato *et al.* 1994, Seiki *et al.* 1994). It has been identified in both a membrane-associated and a shed soluble form in cultured breast cancer cells and fibroblasts as well as in the body's inflammatory exudates, (i.e. bronchoalveolar lavage fluid, gingival crevicular fluid and tear fluid) (Li *et al.* 1998, Maisi *et al.* 2002, Tervahartiala *et al.* 2000, Holopainen *et al.* 2003). It is also expressed by skin fibroblasts (Madlener 1998, Okada *et al.* 1997) and endothelial cells (Silletti *et al.* 2001). Besides activating proMMP-2, -8 and -13, MT-MMPs can also degrade a variety of ECM molecules *in vitro* (Table 1) (Seiki *et al.* 1999, Holopainen *et al.* 2003, Knäuper *et al.* 1996b).

1.2.6. Other MMPs

MMP-19 was cloned from the mammary gland (Cossins *et al.* 1996, Pendas *et al.* 1997). The protein is most similar to the stromelysin class of MMPs in

terms of activity. MMP-19 is expressed in several tissues, including placenta, lung, pancreas, ovary, spleen, intestine, thymus and prostate (Cossins *et al.* 1996, Pendas *et al.* 1997b). In addition, it is expressed by capillary endothelial cells in acutely inflamed synovium, suggesting that MMP-19 can play a role in angiogenesis (Kolb *et al.* 1999). MMP-19 is strongly expressed in tumour cells and their surrounding vasculature in benign breast tumour, whereas progression towards invasive phenotype and neoplastic differentiation lead to disappearance of MMP-19 expression by these cells (Djonov *et al.* 2001). It can also have role in monocyte extravasation and subsequent infiltration to tissue (Locati *et al.* 2002). Human MMP-20 (enamelysin) was first found in odontoblasts, and it is primarily located within newly formed tooth enamel (Bartlett *et al.* 1998). Later it was discovered in human tongue SCC cells, tooth pulp and placenta (Väänänen *et al.* 2001). MMP-20 can degrade amelogenin, the major component of the enamel matrix, laminin-5γ2 chain, aggregan and cartilage oligomeric matrix protein, but not fibrillar type I and II collagens (Table 1). The human MMP-21 was characterized from human placenta cDNA. It is expressed in adult brain, lung, testis, ovary, colon and leukocytes as well as kidney along with several carcinoma cell lines (Ahokas *et al.* 2002).

The human MMP-23 lacks a signal peptide and the cysteine switch. By contrast, it does contain a short N-terminal signal-anchor that localizes MMP-23 to the cell membrane, and therefore represents the third subclass of membrane-bound MMPs (Pei 1999a). MMP-23 is activated and secreted by a single cleavage of an RRRR-motif in the trans-Golgi vesicles (Pei *et al.* 2000). Two copies of the MMP-23 genes are present in the short arm of chromosome 1, in a region that contains two identical genomic regions in a tail-to-tail configuration. This region is thought to contain tumour suppressor genes (Gururajan *et al.* 1998). MMP-23 is mainly expressed in ovary, testis and prostate, which suggests a specialized role in reproduction (Velasco *et al.* 1999).

The MMP-28 (epilysin) is a recently cloned member of the human MMP family (Lohi *et al.* 2001) and is most closely related to MMP-19. MMP-28 is expressed by keratinocytes in the epidermis, and by developing germ cells in the testis. In addition, expression of MMP-28 is detected in the lung, heart, colon, intestine and brain (Lohi *et al.* 2001).

Figure 1. Human MMPs structure based on their domain organization (modified Sternlich and Werb 2001). PRE: predomain, PRO: propeptide, furin site, Zn^{2+}binding site, Fi: fibronectin type II inserts, hinge region, TM: transmembrane domain, Cy: cytoplasmic tail, GPI: glycosylphosphatidylinosit ol domain, SA: signal anchor, CA: cysteine array, Ig-like: immunoglobulin-like domain.

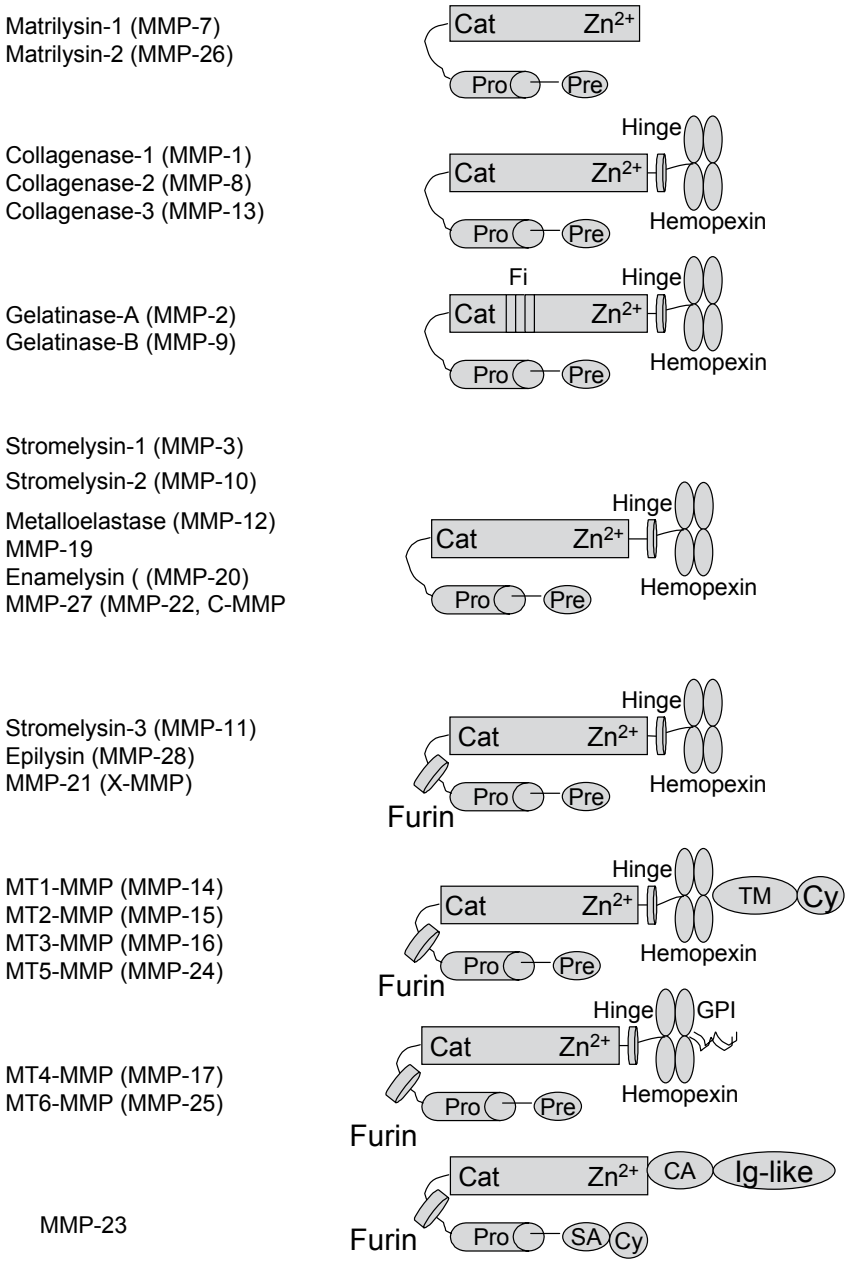


Table 1. Human MMPs and their substrates.

Enzyme	Substrates
MMP-1	(collagenase-1): aggrecan, collagens I-III, VII, X, XI, entactin, FN, gelatin, IGFBPs, Ln-1 link protein, myelibasic, tenascin, α1AC, α2M, α1PI, VN, casein, fibrin, fibrinogen, IL1α and -β, proTNFα, proMMP -1, -2, perlecan
MMP-2	(gelatinase A): aggrecan, collagens I, II-V, VII, X, XI, decorin, elastin, entactin, fibrillin, FN, fibulins, gelatin, IGFBPs, Ln-1, -5, link protein, myelinbasic, osteonectin, tenascin, VN, fibrin, α1AC, α1PI, fibrinogen, IL1β, proTGFβ, proTNFα, plasminogen, substance P
MMP-3	(stromelysin-1): aggrecan, collagens III-V, VII, IX, X, XI, decorin, elastin, entactin, fibrillin, FN, gelatine, IGFBPs, Ln-1, link protein, myelinbasic, osteonectin, tenascin, VN, α1AC, α2M, α1PI, casein, fibrin, fibrinogen, IL1ß, proTNF-α, plasminogen, substance P, E-cadherin
MMP-7	(matrilysin): aggrecan, collagens I-III, entactin, fibrillin, FN, gelatin, Ln-1, -5, VN, α2M, α1PI, FVII, fibrin, fibrinogen, proMMP-2, proTNF-α,
MMP-8	(collagenase-2): aggrecan, collagen I-III, Ln-5, fibrinogen, substance P, α1PI, α2M
MMP-9	(gelatinase B): aggrecan, collagens IV, V, XI, XIV, decorin, elastin, fibrillin, gelatine, Ln-1, link protein, myelin basic, osteonectin, tenascin, VN, α2M, α1PI, casein, fibrin, fibrinogen, IL1β, proTGFβ, proTNFα, plasminogen, substance P
MMP-10	(stromelysin 2): aggrecan, collagens III-V, elastin, FN, gelatine, link protein, casein, fibrinogen, IGFBPs, α2M, α1PI
MMP-11	(stromelysin 3): aggrecan, collagens I, IV, elastin, entactin, fibrillin, FN, gelatine, Ln-1, myelin basic, vitronectin, α2M, α1PI, factor XII, fibrinogen, proTNF-α, plasminogen
MMP-12	(metalloelastase): aggrecan, collagens I, IV, decorin, elastin, entactin, FN, gelatin, Ln-1, link protein, myelin basic, osteonectin, tenascin, VN, α1PI, E-cadherin, fibrinogen, proTNFα, plasminogen, collagen IV, FN, gelatine, α1PI, fibrinogen
MMP-13	(collagenase 3): aggrecan, collagens I-IV,, VI, IX, X, XIV, fibrillin, FN, gelatine, Ln-1, -5, osteonectin, casein, FXII, α2M,fibrinogen
MMP-14	(MT1-MMP): fibrillin, FN, gelatin, proTNFα, collagen IV, FN gelatin, fibrin
MMP-15	(MT2-MMP): collagen 1
MMP-16	(MT3-MMP): collagens I, IV, FN, gelatin, tenascin, casein
MMP-17	(MT4-MMP): gelatin
MMP-18	collagenase 4): gelatin, casein
MMP-19	: collagen II, gelatin, fibronectin
MMP-20	(enamelysin): amelogenin, aggrecan, casein, gelatin, fibronectin, type IV, XVIII collagens, laminin, tenascin C, COMP
MMP-23	: gelatin
MMP-24	(MT5-MMP): proteoglycan, type I collagen, fibronectin, laminin
MMP-25	(MT6-MMP): gelatin, type IV collagen, fibronectin
MMP-26	(matrilysin 2, endometase): fibronectin, fibrinogen, gelatin, type IV collagen, α1PI, laminin-1
MMP-27	: type II collagen, gelatin, fibronectin
MMP-28	(epilysin): casein

Modified from and references from: Van den Steen *et al.* 2002, McCawley and Matrisian 2001, Folgueras *et al.* 2004.

1.2.7. The physiological functions of MMPs

Embryonic growth and tissue morphogenesis are fundamental events that require disruption of ECM barriers to allow cell migration and microenvironmental matrix remodelling. The ability of MMPs to degrade structural components of ECM and BM has supported their direct roles in these processes (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, MMP-7, -3, -10 and -2 are consistently produced during the most active phases of the murine estrous cycle. These MMPs, as well as MMP-8 and MMP-13, 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 analysed 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 generated to date show significant reproductive dysfunction. These 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).

Studies with MMP-9-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 MMP-3-deficient mice show accelerated adipogenesis during mammary gland evolution (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 MMP-3-deficient mice, which exhibit impaired wound contraction (Bullardt *et al.* 1999), and by studies in MMP-1-resistant mice that 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 αvβ3, and this interaction is essential for localizing the enzyme to the surface of newly forming vessels (Brooks *et al.* 1996). Further studies examining the links between MMP-2 and angiogenesis have shown that, after different challenges, MMP-2-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 MMP-2/MMP-9-double deficient mice has demonstrated the synergic involvement 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, -9, -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).

1.3. Regulation of MMPs

In order to avoid unwanted tissue damage it is crucial to accurately control the protease activity. The MMPs are tightly regulated, as they need to be present in the right cell type and pericellular location at the right time and in the right amount. A loss of activity control may result in diseases such as arthritis, cancer, atherosclerosis, aneurysms, nephritis, periodontal disease, tissue ulcers and fibrosis (Kähäri and Saarialho-Kere 1999).

MMPs are regulated both transcriptionally, including signal transduction from cell surface into nucleus, and post-transcriptionally, comprising modulation of mRNA half-life, secretion, localization and activation of proMMPs, as well as inhibition of active MMPs. MMP expression and activity seem only circumscribed under delicate control by their regulatory molecules to those sites and conditions in which proteolytic activity is necessary. However, malignant tumours have developed strategies to circumvent or overcome this regulatory mechanism to generate the uncontrolled and often pathologically excessive proteolytic activity associated with cancer development and metastasis (Nagase and Woessner 1999).

1.3.1. Transcriptional regulation of MMP genes

In normal tissues, MMPs are usually expressed at low levels but their production and activation is rapidly induced when active tissue remodelling or processing is needed. Most MMPs are closely regulated at the level of transcription, with the notable exception of MMP-2, which is often constitutively expressed and controlled through a unique mechanism of enzyme activation (Strongin *et al.* 1995) and some degree of post-transcriptional mRNA stabilization (Overall *et al.* 2001). Furthermore, since they store preconstructed MMP-8 and MMP-9 in specific and secretory granules, respectively, neutrophils can rapidly release or degranulate these MMPs if required (Weiss *et al.* 1989, Ding *et al.* 1997,

1996). In other cells, the expression of MMPs is induced or up-regulated in response to exogenous signals. Because the substrate specificities of distinct MMPs overlap, their biological function may also be determined by differential expression patterns. Otherwise, MMP gene expression is regulated by numerous stimulatory and suppressive factors that influence multiple signaling pathways, for example phorbol esters, integrin derived signals, extracellular matrix proteins, cell stress and contacts, and changes in cell shape (Curran and Murray 2000). MMP expression is regulated by several cytokines and growth factors, including interleukins, interferons, epidermal growth factor (EGF), keratinocyte growth factor (KGF), nervous growth factor (NGF), basic fibroblast growth factor (bFGF), vasculature endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and the extracellular matrix metalloproteinase inducer EMMPRIN (Curran and Murray 2000). These factors are typically released by the stromal cells, infiltrating inflammatory host defence cells or by tumour cells themselves. They activate transcription factors that recognize and bind to specific DNA sequences on the regulatory regions of genes. However, no single factor has been identified that is exclusively responsible for the overexpression of MMPs specific for tumours, although tumour necrosis factor (TNF)-α and interleukin (IL)-1 are often regarded to be implicated (Curran and Murray 2000). The same MMP can be transcriptionally induced or repressed by different agents, depending on the tumour-cell type. Some factors, such as TGF-β or retinoids, function as both positive and negative regulators of MMP transcription in normal and tumour cells (Overall *et al* 1989, Uria *et al* 1998, Overall 1995, Jimenez *et al* 2001).

Extracellular stimuli affect MMP expression via signal transduction pathways like IL-1, TNF-α, PDGF, and EGF that lead to activation of AP-1 (activator protein-1) transcription factors. The expression of AP-1 transcription factors is induced by mitogen-activated protein kinase (MAPK) pathways, extracellular signal-regulated kinase (ERK 1, 2), stress activated proteinase kinase/Jun N-terminal kinase (SAPK/ JNK) and p38. The AP-1 site is in the genes of MMPs-1, -3, -7, -8, -9, -10, -12 and -13 at the proximal promoter approximately at -70 position from the transcription initiation site. MMP-1, -3 and -9 also have another AP-1 site in the distal promoters, but the role of this site is not clear. The MMP-2, -11, -28 and MT1-MMP genes do not have an AP-1 site. AP-1 transcription factors regulate the expression of a variety of genes involved in proliferation, development, differentiation, inflammation, stress response and tumour progression. AP-1 consists of members of the FOS and JUN family of oncoproteins, which could provide a general mechanism for the upregulation of MMP expression in malignant tumours. Several other nuclear factors control MMP expression and are likely to account for the variable inducibility of MMPs by several agents, or even by the same agents in different tumour-cell types. These include the ETS family of oncoproteins, which bind polyoma virus enhancer A binding protein 3 (PEA3) sites that are present in several MMP gene promoters (Curran and Murray 2000). AP-1 and ETS transcription factors can synergistically activate MMP gene transcription (Westermarck *et al*.1997). Their collaboration is also involved in the regulation of genes associated in tumour

progression (Denhardt 1996). Indeed, the expression of ETS-1 colocalizes with the expression of many MMPs at the invading edge of several types of tumours and in tumour vascularization (Bolon *et al.* 1995, 1996, Wernert *et al.* 1994, 1992). Overexpression of ETS transcription factors enhances the activity of MMP-1, -3, and -9 promoters (Buttice *et al.* 1996, Gum *et al.* 1996, Kaya *et al.* 1996). Nuclear factor of κB (NF-κB) induces MMP-1, -3, -9, -13 and -14 (Bond *et al.* 1999, Han *et al.* 2001). Signal transducers and activators of transcription (STATs) mediate the effects of interferons (IFNs) on MMP gene expression (Ala-aho *et al.* 2000). T-cell factor 4 (TCF4) and CAS-associated zinc-finger protein (CIZ) activate the expression of MMP-1, -3 and -7 (Crawford *et al.* 2001, Nakamoto *et al.* 2000). P53 modulates the transcription of MMP-1, -2 and 13 (Sun *et al.* 1999, 2000), and core-binding factor A1 (CBFA1) forms part of a regulatory cascade that controls MMP expression in both normal and tumour cells (Jimenez 2001). Negative regulatory elements, such as TGF-β inhibitory element (TIE) or AG-rich element (AGRE), have also been identified in the promoters of several MMP genes (Kerr 1990, Benderdour 2002). The promoters of MMP-2, MT1-MMP and MMP-28 lack the common TATA boxes, but they contain target sequences for the Sp-1 transcription factor, which is commonly expressed in a variety of tissues and regulating many genes (Suske 1999). In addition, the promoters of MMP-3 and MMP-9 contain Sp-1 binding sites (Huhtala *et al* 1991). The Sp-1 sequence is essential for the upregulation of MMP-9 transcription in cancer cells and in the promoter activity of MT1-MMP (Gum *et al.* 1996, Lohi *et al.* 2000). The promoter region of human MMP-13 contains an osteoblast-specific element 2 (OSE2) that mediates the expression of osteoblastic-specific genes involving bone formation. Therefore, it could be involved in stimulating bone tumours or osteosarcomas (Ducy *et al.* 1997, Jimenez *et al.* 1999).

Single-nucleotide polymorphisms present in the promoter of MMP genes create or abolish transcription-factor binding sites, thereby modifying MMP transcriptional activity. Specific MMP-1 and MMP-3 alleles have been associated with an increased susceptibility to several different types of cancer, including breast and ovarian carcinomas (Rutter *et al.* 1998, Biondi *et al.* 2000).

Post-transcriptional mechanisms also modulate MMP expression. Examples include stabilization of MMP-1 and -13 mRNA transcripts by phorbol esters and EGF as well as stabilization of MMP-2 and -9 mRNA transcripts by TGF-β (van den Steen *et al.* 2002). The turnover of MMP-1 mRNA is apparently regulated by AU-rich sequences in the 3′-untranslated region, and similar sequences may also regulate the stability of other MMP transcripts (Sternlicht and Werb 2001). MMP expression could also be regulated by alternative splicing. Multiple transcripts have been reported for MMP-8, -11, -13, -16, -17, -20, -25 and -26 (Hu *et al.* 1999, Luo *et al.* 2002). Overall, it is clear that the transcriptional regulation of MMP production is a very complicated phenomenon including the regulation of the production and degradation of transcription factors and the regulation of their trans-activating activities and via these processes the modulation of MMP production in cells.

1.3.2. Activation of proMMPs

Most MMPs are secreted as latent, inactive proenzymes or zymogens, and their activity is controlled extracellularly by zymogen activation and inhibition. Cleavage by furin-like serine proteases can intracellularly activate proMMP-11, -28, -23 and MT-MMPs in the Golgi apparatus before secretion as active enzymes (Pei and Weiss 1995). MMPs can be activated by proteinases or *in vitro* by conformational perturbants, such as thiol reagents, organomercurials, heavy metals (Hg and Au), detergents and oxidants (Nagase 1997). Low pH and heat treatment can also lead to activation. Certain serine proteinases, such as plasmin, chymotrypsin, cathepsin, trypsins, neutrophil elastase, kallikrein and mast cell tryptase, other MMPs, as well as bacterial and fungal proteases can also induce the activation pathway by a series of successive and/or single cleavages (Sorsa *et al.* 1992, 1997, Ding *et al.* 1995, Holopainen *et al.* 2003, Moilanen *et al.* 2003). The proMMPs latency is maintained by a cysteine switch, the interaction between cysteine residue and zinc ion. During activation the opening of the Cys-zinc bond allows zinc ion to react with H_2O to maintain the stabilized open form of MMP, after which it still needs to pass through several structural changes to become fully active. MMP-9 has recently been detected to be active in binding to a substrate even its full-size proform, but the activation still needs the disengagement of the propeptide from the enzyme. This activation model is thought to take place via binding to a ligand or substrate (Bannikov *et al.* 2002). *In vivo* such full-size activated MMP-9 species can be detected in periodontitis-affected gingival crevicular fluid (Westerlund *et al.* 1996). In neutrophils, the proMMP-9 forms complex with integrin $\alpha_M\beta_2$ in intracellular granules, but after cellular activation it becomes localized to the cell surface (Stefanidakis *et al.* 2004).

Activation of proMMPs by plasmin is a relevant pathway *in vivo.* Plasmin is generated from plasminogen by tissue plasminogen activator bound to fibrin and by urokinase plasminogen activator bound to a specific cell surface receptor. Both plasminogen and urokinase plasminogen activator are membrane-associated, thereby creating localized proMMP activation and subsequent ECM turnover. Plasmin has been reported to activate proMMP-1, -3, -7, -9, -10 and -13 (Murphy and Grappe 1995).

Activated MMPs can participate in processing other MMPs and interfere with finer regulatory mechanism (Lijnen 2001). MMP-3 is able to activate MMP-1, proMMP-2, MMP-8 and MMP-9. Active MMP-2 and MMP-13 can in collaboration activate proMMP-9, MMP-13 can be activated by MMP-2, MMP-3 and MMP-10 (Knäuper *et al.* 1996b, D'Ortho *et al.* 1997, Cowell *et al.* 1998, Curran and Murray 2000, Deryugina *et al.* 2001, Holopainen *et al.* 2003). MMP-7 can activate proMMP-8 and -9 (Balbin *et al.* 1998, von Bredow *et al.* 1998). Hence it is highly difficult to define the initiator of the activation cascades as well as how the first enzyme in the cascade is activated. One possibility is that some of the proenzymes are sufficiently active of the initial cleavage for the matrix destructive serine proteinase human tumour-associated trypsin-2 to act as the first initial proteolytic activator of proMMP-activation cascade (Sorsa *et al.* 1997, Moilanen *et al.* 2003).

The main activation of proMMP-2 takes place on the cell surface and is mediated by MT-MMPs including MT1-MMP, MT2-MMP, MT3-MMP, MT5-MMP and MT6-MMP. MT4-MMP does not activate proMMP-2 (Visse and Nagase 2003).

MT1-MMP-mediated activation of proMMP-2 has been studied extensively. Activation of MMP-2 by MT1-MMPs involves TIMP-2. The N-terminal domain of TIMP-2 binds to and inhibits MT1-MMP, whereas the C-terminal domain of the same TIMP-2 molecule binds the hemopexin-like domain of MMP-2 forming a ternary complex. An adjacent TIMP-free MT1-MMP subsequently cleaves the MMP-2 to the intermediate 64-kDa form by cleaving the Asn37-Leu38 bond located in a readily accessible bait region between the first and the second α helix of the prodomain. This intermediate form is then processed into the fully mature 62-kDa form through cleavage of the Asn80-Tyr81 bond by an already active MMP-2 molecule (Murphy and Grappe 1995, Strongin *et al.* 1995). Only the active MT1-MMP binds MMP-2 on the cell surface; regulation of MT1-MMP activation is thus an important control point to regulate MMP-2 activity (Lehti *et al.* 1998). Although TIMP-2 is normally required for the MMP-2 activation, higher TIMP-2 levels lead to inhibition of MMP-2 activation. On the other hand, soluble MT1-MMP activates MMP-2 with a high efficiency in the absence of TIMP-2 (Pei and Weiss 1996). ProMMP-2 activation by MT2-MMP is direct and independent of TIMP-2 (Morrison *et al.* 2001). The cell surface activation of MMP-13 by MT1-MMP can be direct or enhanced with active MMP-2 (Knäuper *et al.* 1996b). MMP-8 can also be activated by MT1-MMP (Holopainen *et al.* 2003). Integrins are also involved in the activation process directly or indirectly, as αvβ3 and β_1 integrin activating antibodies modulate MMP-2 activation (Yan *et al.* 2000).The direct involvement is supported by the finding that the intermediate active MMP-2 is capable of $\alpha v\beta_3$ integrin binding, and this interaction could thus affect MMP-2 activation (Brooks *et al.* 1996, Deryugina *et al.* 2001).

1.4. Mechanisms of cancer growth, invasion and metastasis formation

The term cancer describes a heterogeneous group of more than 200 different types of malignant tumours (Clark 1991). In the year 2002, 23,283 people in Finland were diagnosed with cancer and 9,875 died from it in 2001(Finnish Cancer Registry). Mouth and pharynx cancer is the eight most common solid tumour in the world (Parkin *et al.* 1999). In Finland, 456 new cases were diagnosed in 2002, 106 of which were squamous cell carcinomas of the tongue (Finnish Cancer Registry).

Tumour progression is a complex and multistage process in which normal cells undergo genetic alteration, lose their normal proliferative control and become able to invade and colonize surrounding tissue and eventually distant target organs. In most cases, a tumour shows a selective non-random pattern of metastasis to particular organs, depending on the site where the primary tumour occurs (Rusciano and Burger 1994, Fidler 1995).

Benign epithelial tumours have an intact BM that separates the neoplastic epithelium from the stromal connective tissue, whereas malignant epithelial tumours have a defective BM that allows neoplastic cells to invade the underlying stroma (Barsky *et al.* 1983). The initial stage of tumour invasion is the loss of an intact BM. Tumour cell invasion through the BM is thought to be a three-step process. First, the neoplastic cells attach themselves to the underlying BM. Then the malignant cells produce proteolytic enzymes to degrade the BM. Finally, the tumour cells pass through the BM and spread into the adjacent connective tissue. The steps of attachment, degradation and invasion are repeated within the ECM during tumour growth and spread. A malignant tumour spreads to other parts of the body by first invading through the wall of a blood vessel or a lymphatic vessel. The tumour cells then travel with the stream, attach to a distant location and degrade the BM and ECM at the site of the metastasis. The continued growth and survival of solid neoplasms requires angiogenesis, the growth of new blood vessels from pre-existing ones. Without new blood vessels to provide nutrients and remove waste, tumours would be unable to grow larger than 2-3 mm in diameter. Tumour-induced lymphangiogenesis also plays a role in tumour progression (Folkman 1995, Skobe *et al.* 2001).

The current view concerning the role of stromal component in tumour growth points out that stroma actually participates in tumour progression and that stromal fibroblasts and inflammatory cells can cause tumourigenic conversion of epithelial cells. Evidence has also been provided for the interplay between tumour cells and the stroma from findings that in the early stages of epithelial malignancy, when the basement membrane is still intact, angiogenesis is observed in stroma (Hanahan and Folkman 1996). Some studies have confirmed that tumour can generate its own non-malignant stroma and that one function for this is the reciprocal interaction with epithelial tumour cells to facilitate tumour growth (Lewis *et al.* 2004, Petersen *et al.* 2003).

Using an immunocytochemistry assay (ICC), circulating tumour cells or micrometastases have been detected in almost half of head and neck cancer (HNC) patients (Wirtschafter *et al.* 2002). The clinical implications of circulating tumour cells in patients with HNC have not been completely clarified. However, in the case of prostate cancer, the patients with circulating tumour cells have decreased disease-free and overall survival in comparison to patients without circulating cells (Wirtschafter *et al.* 2002).

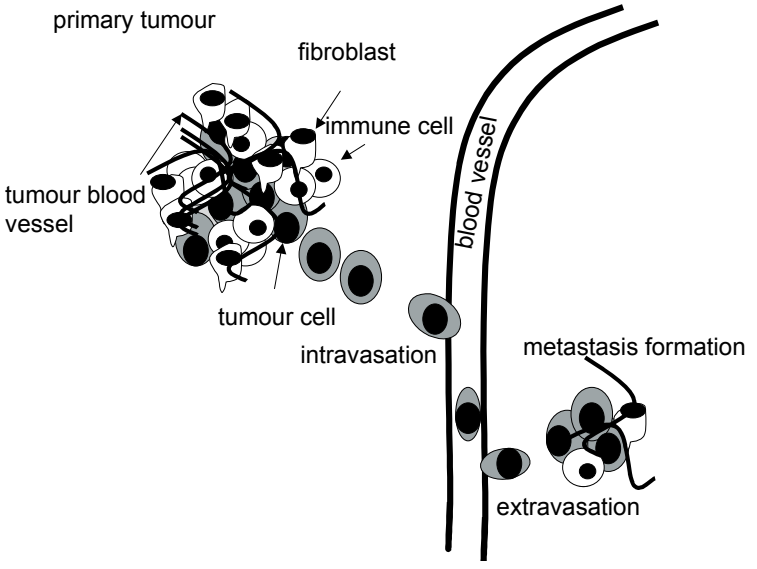
1.4.1. MMPs in cancer

Originally, MMPs were considered to be almost exclusively important in invasion and metastasis of cancers. However, in addition to invasion, current MMP actions are known to contribute to multiple steps of tumour progression, including tumour growth, apoptosis, angiogenesis and spread and growth of metastatic lesions at distant organ sites. MMPs are not only synthesized by tumour cells but are also produced by surrounding stromal cells, including fibroblasts and infiltrating inflammatory cells. In addition to creating gaps in matrix barriers, MMPs can solubilize cell surface and matrix-bound factors that can act in an

autocrine or paracrine manner influencing cell growth, death and migration. In this regard MMPs have both cancer-promoting and cancer-inhibiting as well as anti-inflammatory functions, and pathways with opposing effects on cancer progression are sometimes initiated by cleavage of the same substrate (Balbin *et al.* 2003, Andarawewa *et al.* 2003, Owen *et al.* 2004, Sorsa *et al.* 2004). The proposed roles of MMPs in these processes are based on both *in vitro* and *in vivo* preclinical studies as well as on studies of clinical tissue specimens (Overall and Lopez-Otin 2002).

The development of an altered stromal microenvironment is a common feature of many tumours. There is increasing evidence that these stromal changes, which include increased focal expression of proteases, cytokines and growth factors, may actually promote tumour progression. A common finding is that stromal fibroblasts become activated myofibroblasts, expressing smooth muscle actin and secreting cytokines, growth factors, proteases and matrix proteins. In one study oral SCC cells and primary oral fibroblasts directly induced a myofibroblastic phenotype, and this transdifferentiation was found to be dependent on SCC-derived TGF-β1. In turn, myofibroblasts secrete significantly higher levels of hepatocyte growth factor/scatter factor compared to fibroblast controls, and this factor promotes SCC invasion through Matrigel, a mixture of basement membrane proteins (Lewis *et al.* 2004, Petersen *et al.* 2003).

Figure 2. Schematic representation of the steps in tumour progression. MMPs have been implicated to play a role in most of these steps. Tumours contain in their stromal compartment non-malignant cells such as fibroblasts and immune/inflammatory cells, which may act as a source for MMPs and other molecules to nourish tumour development.



1.4.2. MMPs in tumour growth

There is 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 that are bound to specific binding proteins (IGF-BPs) (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. For example, cell surface-localized MMP-9 proteolytically activates TGF-β promoting tumour growth, invasion and angiogenesis (Yu and Stamenkovic 2000). In contradiction, some investigators showed that MMPs might negatively regulate cancer cell growth by activating TGF-β. One key event that leads to TGF-β-induced growth arrest is the induction of expression of the CDK (cycline dependent kinase) inhibitors (Derynck *et al.* 2001). 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 had no 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 the importance of physical presentation of the surrounding ECM on cell behaviour (Cukierman *et al.* 2001). In MMP-9-deficient mice cancer cell proliferation was decreased in tumours compared with wild type mice (Coussens *et al.* 2000).

1.4.3. MMPs in apoptosis

The ability of MMPs to target substrates that influence the apoptotic process, positively or negatively, 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 (FasL), can also induce 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). Released FasL induces apoptosis of neighbouring cells, or decreases cancer cell apoptosis, depending on the system (Powell *et al.* 1999, Mitsiades *et al.* 2001). In this regard, mice deficient in MMP-2, -3 or -9 have lower levels of apoptosis induced by TNF-α, suggesting 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 by inhibiting cancer cell death (Boulay *et al.* 2001). MMP-11-/- MMTV-ras transgenic mice develop more metastases than their MMP-11+/+ 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 may have another molecular function that leads to a decreased metastatic rate. MMPs are also part of the apoptotic process, for example cleaving E-cadhering and PECAM-1 during apoptosis of endothelial and epithelial cells (Ilan *et al.* 2001, Steinhusen *et al.* 2001). These observations emphasize the importance of selectively targeting certain MMP functions instead of completely blocking their activity.

1.4.4. MMPs in the inflammatory reactions

MMPs have traditionally been associated with the 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 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 are eventually beneficial to the host by stimulating protective and adaptive immune responses. In this regard, a recent report has revealed that mutant male mice deficient in MMP-8 exhibit an increased skin tumour susceptibility compared to wild-type mice (Balbin *et al.* 2003). Furthermore, MMP-8 expression was found to be under hormonal control. Male MMP-8 knock-out mice were more suspectible to cancer than ovaries removed female mice (Balbin *et al.* 2003). Histopathological analysis of these MMP-8-deficient mice has revealed the presence of abnormalities in the inflammatory response induced by carcinogens. In fact, the lack of MMP-8 hampers the early stages of inflammation, but once established it is abnormally sustained leading to a more favourable environment for tumour development. Inflammatory cells, such as macrophages, neutrophils and mast cells, of a developing neoplasm facilitate genomic instability, promote angiogenesis and produce chemokines and cytokines that induce or inhibit MMP transcription or activation and can influence tumour development and its microenvironment (Coussens and Werb 2001, 2002). Therefore, and contrary to previous studies performed with mice lacking specific MMPs, loss of MMP-8 enhances rather than reduces tumour susceptibility (Balbin *et al.* 2003). A putative mechanism to explain these paradoxical or unexpected effects of a member of the MMP family comes from its potential proteolytic processing activity on inflammatory mediators, which could contribute to the host antitumour defence system (Coussens and Werb 2002).

1.4.5. MMPs in angiogenesis

The role of MMPs in angiogenesis is considered to be dual and complex. The relevance of MMPs as positive regulators of tumour angiogenesis has been largely demonstrated. Thus, several pro-angiogenic factors such as VEGF, bFGF or TGF-β are induced or activated by MMPs, 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). MMPs might simply act by degrading the ECM, which would allow endothelial cells to invade the tumour stroma (Seandel *et al.* 2001). An additional connection between angiogenic factors and MMPs derives from the recent finding that MMP-9 is induced in tumour macrophages and endothelial cells to promote lung metastasis (Hiratsuka *et al.*

2002). Furthermore, 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 MMPs can negatively regulate angiogenesis have contributed to enhance 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 angiogenesis inhibitors (Cornelius *et al.* 1998, Ferreras *et al.* 2000, Heljasvaara *et al.* 2005). 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).

1.4.6. MMPs in invasion and metastasis

The importance of MMPs in cancer cell invasion originated from the study by Liotta *et al.*, who were the first to show that metastatic properties of tumour cells correlate with the degradation of type IV collagen, the main collagenous component of the BM (Liotta *et al.* 1980).

Overexpression of MMP-2, -3, -13 and -14 promotes, while overexpression of TIMPs inhibits the invasion of cancer cell lines through either collagen I, optic nerve explants or Matrigel (Ahonen *et al.* 1998, Lochter *et al.* 1997, Belien *et al.* 1999, Deryugina *et al.* 1997, Ala-aho *et al.* 2002). In experimental metastasis assays, the number of colonies formed in the lungs of mice is reduced by MMP-9 downregulation in cancer cells, and is also reduced in the MMP-2 and -9-null mice as compared with wild type mice (Hua and Muschel 1996, Itoh *et al.* 1998, Itoh *et al.* 1999). MMP-2, -14 and -13 can degrade laminin-5γ2-chain, another BM component, which plays an important role in epithelial cell motility (Giannelli *et al.* 1997, Koshikawa *et al.* 2000, Pirilä *et al.* 2003). The interaction of laminin-5 with α6β4 integrin leads to the assembly of hemidesmosomes that anchor epithelial cells to the underlying basement membranes (Baker *et al.* 1996). Epithelial cells may be stimulated to express MT1-MMP by environmental signals leading to activation of MMP-2 and cleavage of laminin-5γ2-chain further triggering epithelial cell migration through reconstituted basement membrane (Giannelli *et al.* 1997, Koshikawa *et al.* 2000, Pirilä *et al.* 2003).

Interactions between tumour cells and matrix components are important for the growth and invasion of malignant tumours (Iozzo, 1995). During migration, which is the first step in invasion, cancer cells must detach from both neighbouring cells and the surrounding matrix. Thus, migration is regulated by cycles of localized MMP activity, rather than by continuously high MMP activity.

CD44 is a major hyaluronan receptor that is involved in cell-cell and cell-matrix interactions. CD44 is cleaved by MMP-14 and the extracellular domain is released to stimulate migration of the pancreatic tumour cell line (Kajita *et al.* 2001). In addition to binding to the ECM, CD44 also binds MMP-9, thereby augmenting the enzyme localization to the cell surface. This localization is required for MMP-9 to promote tumour invasion and angiogenesis. CD44,

MMP-2, -9 and -14 are reported to be found at the edge of the motile cells, on specialized surface protrusions, called invadopodia (Nakahara *et al.* 1997, Bourguignon *et al.* 1998). The epithelial cell-cell adhesion molecule, E-cadherin, can be cleaved by MMP-3 and -7 (Noe *et al.* 2001). The released fragment of E-cadherin promotes tumour cell invasion in a paracrine manner *in vitro*, by binding to and interfering with the function of other full-length E-cadherin molecules (Noe *et al.* 2001). Cleavage of E-cadherin transcriptionally affects the epithelial to mesenchymal -transition as well (Birchmeier *et al.* 1996).

Integrins, a family of heterodimeric cell adhesion molecules composed of an α chain and a β chain, are one of the key players in the regulation of cell migration. Various α and β chains combinations bind to the specific cell surface and ECM ligand transmitting signals between the outside and the inside of the cells (Giancotti and Ruoslahti 1999, Hynes 2002). MT1-MMP and MT3-MMP have been found to be linked to proteolytic cleavage of FAK in vascular smooth muscle cells. The FAK receptor can be activated through integrin- mediated signals and it can regulate multiple functions such as cell motility, survival and proliferation (Shofuda *et al.* 2004).

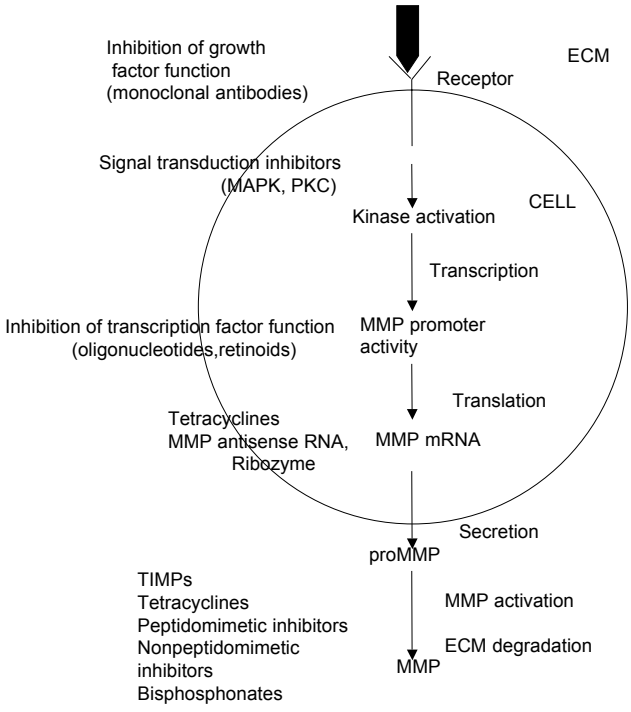
A possible complication in cancer therapy with cell migration inhibiting agents is that the migration mechanisms utilized by the cancerous cells and non-neoplastic cells are highly similar or identical. Migration of non-neoplastic cells is required for example in embryogenesis, inflammation and wound healing. Hence, targeted complete inhibition of these processes eventually leads to undesired detrimental side-effects (Friedl and Brocker 2000, Lauffenburger and Horwitz, 1996).

Cancer dissemination to distant organs through blood or lymphatic vessels, penetrating the vascular wall, is a crucial event in metastasis. MMPs can also participate in these late events, when the cancer cells must enter, survive and exit the blood vessels or lymphatics. MMP-9 is required for intravasation (Kim *et al.* 1998). MMP-14 overexpression increases the number of cancer cells that survive intravenous injection in an experimental metastasis assay (Tsunezuka *et al.* 1996). By contrast, MMP activity might not be important for extravasation, as TIMP-1 overexpression cancer cell exit the vasculature equally well as control cells (Koop *et al.* 1994). Metastasis growth thus probably also involves MMP activity. Furthermore, tissue-specific differences may exist among the metastasis-promoting proteinase cascades.

1.5. Matrix metalloproteinase inhibitors

MMPIs could alternatively inhibit or down-regulate MMPs both transcriptionally, including signal transduction from cell surface into nucleus, and post-transcriptionally comprising modulation of mRNA half-life, secretion, localization and activation of proMMPs, and inhibition of active MMPs levels (Westermarck and Kähäri 1999). They can be divided in endogenous MMPIs, such as TIMPs, inhibitors of MMP synthesis including cytokines, signal transduction and transcriptional factors, inhibitors of proMMP activators such as anti-MT1-MMP anibodies and inhibitors influencing the catalytic site of MMPs, a group in which most synthetic MMPIs belong to (Fig. 3).

Figure. 3. Schematic representation of the possible inhibition levels of MMP therapeutic interventions.



1.5.1. Endogenous MMPIs

Two major endogenous inhibitors are known for MMPs, the non-specific or general inhibitors, such as α2-macroglobulin (α2M), which are present in the plasma and tissue fluids, and the more specific inhibitors, called tissue inhibitors of metalloproteinases (TIMPs). Four human TIMPs have been identified, which are anchored in the ECM or secreted extracellularly. They bind MMPs tightly and non-covalently in 1:1 stoichiometric complexes (Brew *et al.* 2001). They differ in tissue-specific expression and ability to inhibit various MMPs. 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). There are numerous examples in which TIMP expression is increased during tumour progression, which could be expected to reduce tumourigenic potential. This may represent a host-protective stromal response. TIMP-4 can up-regulate the anti-apoptotic protein Bcl-X, thereby stimulating mammary tumourigenesis (Jiang *et al.* 2001), whereas TIMP-2 shows cell-growth promoting activity (Baker *et al.* 2002, Jiang *et al.* 2002), indicating that their role *in vivo* is complex, extending the MMP inhibition. The expectation that malignant tumours have increased MMP expression accompanied by decreased TIMP expression is probably too simplistic. However, the possibility of using TIMPs in cancer therapy is limited, since protein-based treatments are difficult to administer and generally suffer from poor pharmacokinetics. Furthermore, TIMPs inhibit a broad spectrum

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 or the protective MMPs (Balbin *et al.* 2003, Owen *et al.* 2004, Sorsa *et al.* 2004). There are also MMP inhibitors that contain subdomains with structural similarity to the TIMPs. These include the procollagen C-terminal proteinase enhancer that releases a C-terminal fragment possessing significant MMP inhibitory activity as well as the NC1 domains of type IV collagen and tissue-factor pathway-inhibitor-2, a serine proteinase inhibitor that can also function as an MMP inhibitor (Mott *et al.* 2000, Netzer *et al.* 1998). RECK (reversion-inducing cysteine-rich protein with kazal motifs) is the only known membrane-bound MMP inhibitor and key regulator of ECM integrity and angiogenesis (Oh *et al.* 2001). At present, the physiological targets of these putative MMP inhibitory activities remain unclear, as does their relevance in cancer.

1.5.2. Inhibitors of MMP synthesis

There are three main approaches for targeting MMP gene transcription: (i) preventing the action of extracellular factors, (ii) blocking signal-transduction pathways, and (iii) targeting those nuclear factors that enhance the expression of the corresponding MMP gene (Westermarck and Kähäri 1999). The diversity of extracellular factors that can mediate MMP production as well as the opposite effects of these factors on the expression of different MMP genes, makes it difficult to select targets. However, recent studies have shown that factors such as IFN-α, IFN-β and IFN-γ can be used to inhibit the transcription of several MMPs by tumour cells (Kuga *et al.* 2003, Ma *et al.* 2001, Slaton *et al.* 2001). Monoclonal antibodies against TNF-α or soluble forms of the TNF receptor are effective in treating rheumatoid arthritis. This might possibly be a potential tool in cancer treatment as well (Mengshold *et al.* 2002). Similar strategies to block IL-1 or EGF receptors might also be useful for abolishing MMP production in cancer (Lal *et al.* 2002). Retinoids and TGF-β have been reported to down-regulate the expression of MMPs and increase TIMP expression; however, other studies have reported the opposite (Overall *et al.* 1989, Uria *et al.* 1998, Overall 1995, Jimenez *et al.* 2001).

A second way to target the blocking of MMP production is the signal-transduction pathways that mediate induction of these enzymes. The blockade of specific steps in the MAPK pathway can lead to the suppression of MMP gene expression in diverse cancer cells. Selective inhibition of p38 MAPK activity by SB203580 abolishes the expression of MMP-1, -9 and -13 by transformed keratinocytes and SCC cells, and is accompanied by reduced invasion through collagen and artificial basement membranes (Johansson *et al.* 2000, Simon *et al.* 1998). The specific inhibitor (SP600125) of the ERK and JNK pathway has led to MMP down-regulation by tumour cells (Shin *et al.* 2002). Other compounds such as halofuginone an alkaloid from the medicinal plant *Dichroa febrifuga,* manumycin A and malolactomycin D can also block MMP gene expression through interference with the TGF-β or Ras signalling pathways (Futamura *et al.* 2001, McGaha *et al.* 2002, Zhang *et al.* 2002).

A third way to inhibit MMP production in human tumours is to target the nuclear factors directly responsible for MMP transcriptional upregulation. Epidemiological studies have suggested that certain dietary factors may act as anticarcinogenic agents, and animal models of carcinogenesis have demonstrated that perhaps as many as 500 agents may have some efficacy as chemopreventive agents, for example curcumin, natural products of the Indian spice turmeric, nobiletin, a flavonoid that is obtained from *Citrus depressa*, resveratrol, a phytoalexin present in grapes and dykellic acid, a fungal metabolite blocking the activity of transcription factors. Glucocorticoids, synthetic terpenoids and NSAIDs (non-steroidal anti-inflammatory drugs) block the activity of transcription factors such as AP1 and NF-κB, which regulate the transcription of several MMP genes (Hasina *et al.* 2001, Karin and Chang 2001, Sato *et al.* 2002, Mix *et al.* 2001, Shishodia *et al.* 2003, Takada and Aggarwal 2003, Aggarwal *et al.* 2003, Banerji *et al.* 2004, Woo *et al.* 2003a, Woo *et al.* 2003b). However, in addition to MMP genes these strategies also affect the expression of multiple genes and they may have several undesired 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).

Similarly, restoring the activity of transcription factors such as p53, PTEN (phosphatase and tensin homologue) and TEL (translocation-ETS-leukaemia), which negatively regulate MMP expression and whose activity is lost in human tumours, could downregulate these genes (Ala-aho *et al.* 2002, Fenrick *et al.* 2000, Koul *et al.* 2001). It is also possible to target MMP synthesis using transfecting cells with antisense mRNA or oligonucleotides, or by targeting mRNA with ribozymes, which are RNA molecules that function like enzymes and exert a catalytic activity. Ribozymes can be designed to cleave specific mRNAs and thereby to inhibit protein synthesis (Kondraganti *et al.* 2000, London *et al.* 2003, Ala-aho *et al.* 2004). Even though there are many opportunities to block the production of MMPs, none of them have yet been translated into clinical applications.

1.5.3. Inhibitors of proMMP activation

There are several new possibilities of MMP inhibition based on targeting proMMP activation. MT1-MMP is a activator of other proMMPs (-2, -8 and -13) and has a pivotal role in regulating tumour growth (Knäuper *et al.* 1996b, Holopainen *et al.* 2003). Anti-MT1-MMP monoclonal antibodies inhibit its proteolytic activity and impair endothelial cell migration and invasion of collagen and fibrin gels, and it could thus be a future anti-cancer drug (Galvez *et al.* 2001). Catechins in greentea and red wine polyphenolic compounds block the MT1-MMP-dependent activation of proMMPs (Annabi *et al.* 2002, Oak *et al.* 2004). Furthermore, the complexity of the enzymatic cascade of MMP activation provides new possibilities to target tumour MMPs by blocking the upstream proMMP-activators such as proMT-MMPs and tumour-associated trypsin-2 (Sorsa *et al* 1997, Paju *et al.* 2001, Moilanen *et al.* 2003, Nyberg *et al.* 2003, Holopainen *et al.* 2003). In this regard, a selective furin inhibitor, α1-PDX prevents MT1-MMP activation and proMMP-2 processing, with the subsequent

attenuation of tumourigenity and invasiveness of human cancer cells (Bassi *et al.* 2001). Similar results have been shown using a synthetic furin inhibitor (Maquoi *et al.* 1998). However, it has not shown any inhibitory effect on the activation of the secreted convertase-sensitive MMPs. IL-4 and IL-13, anti-inflammatory cytokines, inhibit MMPs via the proMMP activation process (van Lent *et al.* 2002). Endostatin is a collagen XVIII-derived 20-kDa proteolytic fragment with anti-angiogenic and anti-tumour properties (Kim *et al.* 2000). It acts as an inhibitor of MMP-2 activation (Kim *et al.* 2000) as well that of MMP-9 and MMP-13 (Nyberg *et al.* 2003). Proteoglycans such as testican-3 and N-Tes can suppress proMMP-2 activation mediated by MT1-MMP (Nakada *et al.* 2001). Furthermore human immunodeficiency virus (HIV) aspartyl protease inhibitor blocks proMMP-2 activation, thereby contributing to the regression of these highly aggressive tumours (Sgadari *et al.* 2002).

1.5.4. Synthetic MMP inhibitors

Most of the synthetic MMP inhibitors target the catalytic site of the MMPs and act by chelating the catalytically essential zinc ion. Due to the huge interest in the therapeutic intervention of MMPs in cancer, over a hundred small-molecule MMP inhibitors have been designed and synthesized (Whittaker *et al.* 1999). The first series of synthetic inhibitors were pseudopeptide derivatives that were synthesized to mimic the structure collagen at the site where MMP binds to cleave it, called peptidomimetic MMP inhibitors. Hydroxyamate inhibitors bind reversibly at the active site of the MMP in a stereospecific manner and chelate the zinc atom on the enzyme activation site (Betz *et al.* 1997). Batimastat (BB-94) is a low-molecular-weight broad-spectrum hydroxamate-based inhibitor that inhibits MMPs and members of the adamalysin family of metalloproteinases (Beattie and Smyth 1998). A recent study showed that batimastat promoted liver metastasis in a mouse model (Kruger *et al* 2001), and it showed no significant responses in clinical trials. It was replaced by marimastat (BB-2516), a low-molecular-weight orally bioavailable MMP inhibitor. It is relatively non-specific inhibiting the activity of MMP-1, -2, -7, -9, -12, -13 and poorly that of MMP-3. The drug contains a collagen-mimicking hydroxamate structure that chelates the zinc ion at the active site of MMPs (Wojtowicz-Praga *et al.* 1998).

Non-peptidic MMPIs have been synthesized on the basis of the three-dimensional conformation of the MMP zinc-binding site (Hidalgo and Eckhardt 2001). They are more specific and have better oral bioavailability than peptidomimetic inhibitors. BAY 12-9566 is an orally bioavailable synthetic biphenyl compound which is a potent inhibitor of MMP-2, -3, -9, and -13 (Rowinsky 2000). Prinomastat (AG3340) is a synthetic, low-molecular-weight collagen-mimicking MMP inhibitor. It is lipophilic and inhibits the activity of MMP-1, -2, -3, -7, -9 and -14 as well as tumour growth and angiogenesis in several xenograft models (Hidalgo and Eckhardt 2001). BMS-275291 is an orally bioavailable inhibitor of MMP-2 and MMP-9, which inhibits tumour growth and angiogenesis. It does not inhibit TNF-RII shedding and thus does not cause joint pain as a side effect (Rizvi *et al.* 2004). MMI 270B/CGS27023A is a non-peptidic MMPI and a potent inhibitor of MMP-1, MMP-2 and MMP-3 activity (Levitt *et al.* 2001). MMI-

166, a selective matrix metalloproteinase inhibitor for MMP-2 and -9, suppressed experimental metastasis of human cancers (Katori *et al.* 2002).

1.5.5. Neovastat

Neovastat is an orally bioavailable extract from shark cartilage. The function of neovastat is based on multifunctional antiangiogenic effects. Neovastat can inhibit the activity of elastase, MMP-2, MMP-9, MMP-12 and MMP-13. Neovastat also inhibits the function of VEGF receptor-2 and induces endothelial cell apoptosis (Gingras *et al.* 2003). The antitumour activity of neovastat has been demonstrated in experimental mouse models with human glioblastoma cell graft, Lewis lung carcinoma, as well as in melanoma, colon and breast cancer models (Gingras *et al.* 2003).

1.5.6. Tetracycline derivatives

Tetracyclines, having antimicrobial effects, were already discovered 60 years ago, but it was only shown much later that tetracyclines and their derivative doxycycline as well as several modified analogues called chemically modified tetracyclines (CMT) inhibit MMP activity independently of their antimicrobial action (Golub *et al.* 1992, 1998, Sorsa *et al.* 1994). Doxycycline and CMT-1 inhibit MMPs with different efficiencies; at therapeutically attainable concentrations doxycycline and CMT-1 inhibit MMP-8, -9, -13 and -14 more efficiently in comparison to MMP-1 and -2 (Suomalainen *et al.* 1992, Greenwald *et al.* 1999, Lee *et al.* 2001, Golub *et al.* 1995).

Metastat (Col-3, CMT-3) is a synthetic MMPI with limited systemic toxicity which belongs to non-antimicrobial CMTs (Lokeshwar *et al.* 2001). Metastat inhibits activated MMPs by interacting with the Ca^{2+} and Zn^{2+} binding sites and prevents activation caused by oxidation (Sorsa *et al.* 1998). Metastat inhibits the activity of MMP-1, MMP-2, MMP-8, MMP-9, MMP-13 and elastase and it downregulates the expression of various inflammatory cytokines. In preclinical studies, metastat inhibited tumour cell proliferation, tumour growth and malignant cell invasion into normal lung tissue in rodent models of metastasis and it also inhibited tumour-induced angiogenesis and induced apoptosis (Hidalgo and Eckhardt 2001, Lokeshwar *et al.* 2002). In addition to MMPs, metastat also affect other proteases, such as human tumour-associated trypsinogen-2 (Lukkonen *et al.* 2000).

1.5.7. Bisphosphonates

Bisphosphonates are synthetic compounds characterized by a P-C-P group. They are used to inhibit bone resorption in diseases like osteoporosis and Paget's disease. Bisphosphonates have also recently become important in the management of cancer-induced bone disease (Brown *et al.* 2004). These effects mainly consist of inhibition of bone resorption and, when given in large amounts, inhibition of ectopic and normal calcification. While the latter effect is the consequence of a physical-chemical inhibition of calcium phosphate crystal formation, the former is due to a cellular effect involving both apoptosis of

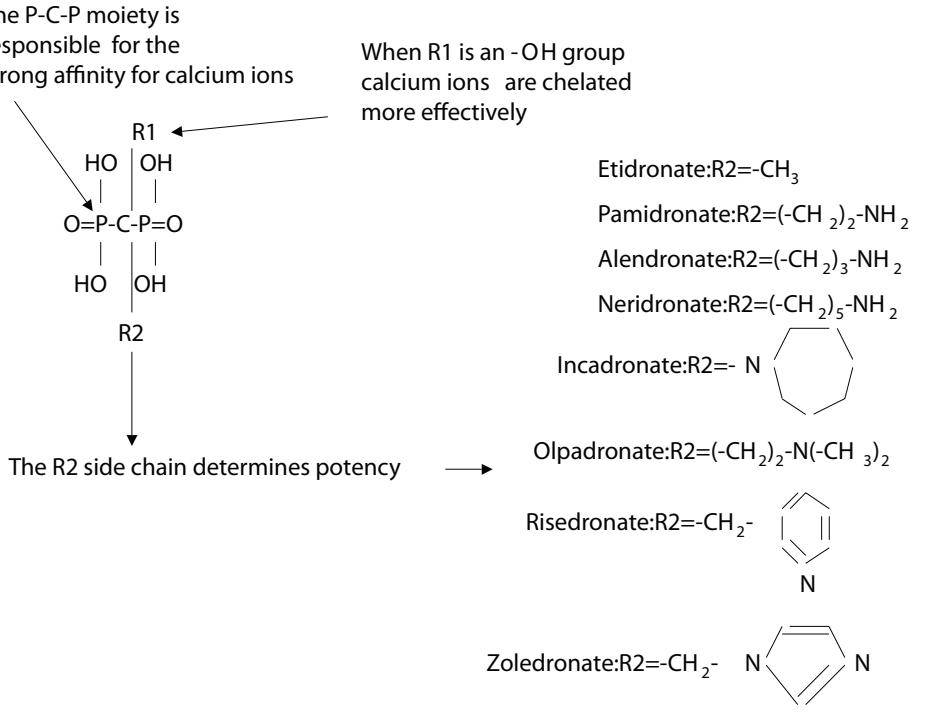
the osteoclasts and a destruction of the osteoclastic cytoskeleton, inducing a decrease in osteoclast activity. The bond of bisphosphonates is stable in heat and most chemical reagents and completely resistant to enzymatic hydrolysis, but can be hydrolyzed in solution by ultraviolet light. Bisphosphonates are generally well tolerated and have low toxicity. However, patients who had received chronic bisphosphonate therapy had necrotic lesions in the jaw; the typical presentation is a non-healing tooth-extraction socket or exposed jawbone (Ruggiero *et al.* 2004). Many bisphosphonates have been investigated in humans with respect to their effects on bone, and eight of them are commercially available today for treatment of bone disease (Fleisch, 2002).

1.5.7.1. Pharmacology of bisphosphonates

The two phosphonate groups of bisphosphonates are linked to the central carbon atom by highly hydrolysis-resistant phosphoether bonds. The central carbon atom can form two additional covalent bonds, and resulting side chains are usually indicated as R1 and R2 (Fig. 4). The P-C-P moiety of bisphosphonates is responsible for their strong affinity for divalent metal ions, such as calcium ions, and for the skeleton. Furthermore, when the R1 side chain is a hydroxyl group, such compounds are able to chelate calcium ions more effectively, by tridentate rather than bidentate binding. An up to 1000-fold increase in potency was achieved by the introduction of a primary amino group (-NH2) at the extremity of the R2 alkyl chain to form the amino-bisphosphonates (e.g. alendronate, pamidronate and neridronate). Amino-bisphosphonates with a secondary amino group (e.g. incadronate) and a tertiary amino group (e.g. olpadronate) are even more effective, and potency reaches its peak when a tertiary nitrogen is included within a ring structure in the R2 side chain (as in risedronate and zoledronic acid) (Russell and Rogers 1999).

Currently, the molecular mechanism(s) through which bisphosphonates exert their activity is only beginning to be understood. The bisphosphonates can be classified into two major groups with different modes of action. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway, the main target being farnesyl diphosphate synthase (Brown *et al.* 2004). Inhibition of the mevalonate pathway leads to loss of important prenylated proteins which are required for post-translation lipid modification (i.e. prenylation) of signaling GTPases, such as Ras, Rho and Rac (Rogers *et al.* 2000). These regulate a variety of key osteoclast cell functions, such as control of endosomes, integrin signaling, membrane ruffling and control of cell morphology, and loss of these proteins leads to induction of osteoclast apoptosis. Non-nitrogen-containing bisphosphonates have a different mechanism of action. They are metabolically incorporated into non-hydrolysable analogues of ATP, ultimately also leading to osteoclast apoptosis. As a consequence of this osteolysis is effectively inhibited. These metabolic reactions are likely to be catalysed by members of the family of type 2 class of aminoacyl-tRNA synthetases, which play a pivotal role in protein synthesis (Rogers *et al.* 1996).

Figure 4. Bisphosphonate chemical structure. The two phosphonate groups are linked to the central carbon atom by phosphoether bonds. The carbon atom forms two additional covalent bonds, and the resulting side chains are referred to as R1 and R2.



1.5.7.2. Anti-tumour effects of bisphosphonates

Increasing evidence is accumulating *in vitro* and *in vivo* that bisphosphonates are able to directly affect tumour cells by mechanisms such as apoptosis induction, inhibition of cell growth, inhibition of invasive behaviour and inhibition of angiogenic factors, in addition to their direct effects upon osteoclasts. The potency of the anti-tumour effect *in vitro* generally mirrors the potency of the anti-resorptive ability with nitrogen-bisphosphonates, in particular, zoledronic acid being the most potent in both respects.

Bisphosphonates can induce apoptosis of tumour cells and inhibit tumour cell growth of a variety of tumour cell types *in vitro* (Fromigue *et al.* 2000, Senaratne *et al.* 2000, Jagdev *et al.* 2001a, Lee *et al.* 2001, Riebeling *et al.* 2002, Mackie *et al.* 2001, Sonnemann *et al.* 2001, Cheng *et al.* 2004, Shipman *et al.* 1998). These results are consistent with earlier reports that bisphosphonates can inhibit cell proliferation and induce apoptosis in osteoclast (Ito *et al.* 1999) and in J774 macrophage-like cells (Selander *et al.* 1996). Nitrogen-bisphosphonates pretreatment of prostate and breast carcinoma cell lines has been shown to inhibit tumour cell adhesion and spreading in parallel to their antiresorptive potency to

unmineralized and mineralized bone extracellular matrices in a dose-dependent manner, whereas non-nitrogen-bisphosphonates have little or no effect (van der Pluijm *et al.* 1996, Boissier *et al.* 1997). Furthermore bisphosphonate recognition by tumour cells did not modulate cell surface integrin expression (Boissier *et al.* 1997). The growth inhibition of breast cancer cells induced by both bisphosphonate groups is augmented by p38 MAPK pathway inhibition *in vitro* (Merrell *et al.* 2003).

MMPs, important for normal and malignant remodelling, evidently contribute to bone metastases. Bone marrow stromal cells from myeloma patients secrete MMP-1 and MMP-2. MMP-1 initiates bone resorption by degrading type I collagen, which becomes a substrate for and can also be directly degraded by MMP-2 (Barille *et al.* 1997, Konttinen *et al.* 1998). Malignant plasma cells have been found to upregulate MMP-1, MMP-8 and MMP-13 and to activate MMP-2 (Derenne *et al.* 1999, Wahlgren *et al.* 2001). The bisphosphonates exert varying inhibitory effects on MMPs, including inhibition of their enzymatic activity; the ability of bisphosphonates to act as direct inhibitors of the activity of MMPs was originally discovered by our group (Teronen *et al.* 1997a, 1997b, 1998, 1999, 2001). Many studies have thereafter provided evidence for a direct cellular effect of bisphosphonates in preventing tumour cell invasion and an inhibitory effect of bisphosphonates on the proteolytic activity of MMPs (Montague *et al.* 2004). In osteoblasts, alendronate at concentrations higher than 10 μM markedly stimulated the synthesis of MMP-13 mRNA and immunoreactive protein, but did not stimulate the transcriptional rate of the MMP-13 gene. Alendronate did not alter the expression of TIMP-1 and -2, but modestly stimulated the expression of TIMP-3. The actions of alendronate in osteoblasts suggest potential additional effects in bone remodelling (Varghese and Canalis, 2000).

When tumour cells were injected into the bone medullary cavity of SCID mice femurs both *in vivo* and following isolation *in vitro*, the levels of MMP-2 and MMP-9 secreted in the bone medullary cavity of the femurs directly correlated with the extent of collagen I release, while alendronate pretreatment *in vivo* blocked both MMP production by the tumour cells and the release of osteoclasts and collagen I (Stearns and Wang 1998). Clodronate can directly inhibit the activities of MMP-1 and MMP-8 *in vitro* (Teronen *et al.* 1997a, b). Alendronate inhibited the secretion of MMP-2, and it prevented the inhibitory effect of TIMP-2 on MMP-2 degradation by plasmin and thereby enhances inactivation of MMP-2 (Farina *et al.* 1998, Stearns *et al.* 1998, Cheng *et al.* 2004). Zoledronate inhibited both IL-6 and MMP-1 production in bone marrow stromal cells, but up-regulated MMP-2 secretion from bone marrow stromal cells (Derenne *et al.* 1999).

Bisphosphonates have anti-angiogenic effects as well. Osteoblastic cells in bone marrow produce both VEGF and b-FGF, and vascularization is needed for osteoclastic bone resorption. Intravenous zoledronic acid or pamidronate, given as treatment for metastatic bone disease to breast cancer patients, decreased bone marrow plasma values of b-FGF and VEGF (Jagdev *et al* 2001b), while pamidronate decreased serum VEGF of cancer patients with a variety of solid tumours (including non-small cell lung cancer, breast, prostate and bladder

cancers) that had metastasized to bone (Santini *et al.* 2002). Zoledronic acid and pamidronate have also been shown to decrease b-FGF- and, to a lesser extent, VEGF-induced proliferation of vascular tissue in a murine soft tissue model of angiogenesis (Wood *et al.* 2002).

In vitro, bisphosphonates can enhance the anti-tumour activity of known cytotoxic agents that are commonly used in the clinical setting, but very little is known about how bisphosphonates interact with commonly used cytotoxic agents (Magnetto *et al.* 1999). Zoledronic acid has shown synergy with paclitaxel *in vitro* in breast cancer treatment (Jagdev *et al.* 2001a) and with dexamethasone in myeloma (Tassone *et al.* 2000). Other bisphosphonates in combination with cytotoxic drugs have at best shown additive activity, e.g. ibandronate in combination with taxanes in breast cancer (Magnetto *et al.* 1999), or no effect, e.g. pamidronate in combination with the chemotherapy agent dacarbazine (Riebeling *et al.* 2002). The new bisphosphonate minodronate (YM529) with the chemotherapy agent etoposide further inhibited the production of bone metastasis and significantly prolonged survival of human small-cell lung cancer-bearing mice (Yano *et al.* 2003). Furthermore, the nitrogen-bisphosphonates activated the human γδT cell population, which shows potential cytotoxic activity toward a broad spectrum of tumours (Kunzmann *et al.* 1999, 2000).

Bisphosphonates reduce tumour osteolysis and bone destruction and prolong survival in animal tumour models. The 4T1 orthotopic murine mammary tumour cell model, where cells are injected into the mammary fat pad, mimics the human situation. Zoledronic acid not only led to a reduction in new bone metastases and osteolysis, but interestingly also inhibited progression of excisting lesions. Non-osseous tumour was however not affected (Mundy *et al.* 2001). Mice receiving both ibandronate and TIMP-2 transfected breast cancer cells exhibited no radiologically detectable osteolytic lesions (Yoneda *et al.* 1997).

Other bisphosphonates in similar animal models have either shown no effect (Krempien and Manegold 1993) or an adverse effect (Kostenuik *et al.* 1993). In a model of breast cancer bone metastases, risedronate inhibited the development of bone metastases, and for those mice receiving continuous risedronate, survival was increased compared with untreated mice. However, while risedronate-treated animals had a significant decrease in bone tumour load, there was a greater amount of metastatic invasion into soft tissues surrounding bone when compared with the untreated animals. In contrast, the experimental bisphosphonate YH529, when started the same day as MDA-MB-231 breast cancer cell inoculation into nude mice reduced non-osseous metastases, in addition to the inhibition of bone lesions and tumour burden in bone (Sasaki *et al.* 1998).

1.5.7.3. Bisphosphonates in anti-cancer therapy

Bone is a preferred site of metastasis for many solid tumours, and the complications associated with bone metastases can result in significant skeletal complications, including pathologic fractures, bone pain, impaired mobility, spinal cord compression and hypercalcaemia. Bisphosphonates are the current standard of care for preventing skeletal complications associated with bone

metastases inducing a diminution of bone resorption, leading to a decrease in hypercalcaemia, new osteolytic lesions and fractures, and leading to an amelioration of pain and an improvement of the quality of life (Fleisch 2002). In addition, bisphosphonates may prevent cancer-treatment-induced bone loss (CTIBL) and the development of malignant bone disease in patients with early-stage cancer (Lipton 2004). Additionally, there is increasing evidence to support the adjuvant use of bisphosphonates in breast cancer, and confirmatory studies are in progress (Lipton 2004).

Bone metastases are common in patients with many types of cancer, especially breast and prostate cancer. Patients with multiple myeloma, lung cancer, bladder cancer, renal cell carcinoma, thyroid cancer and melanoma are also at high risk for skeletal complications (Coleman 2004). Patients who receive adjuvant hormonal therapy for breast cancer or androgen-deprivation therapy for prostate cancer are at an especially high risk for CTIBL because of reduced oestrogen signalling. Oral clodronate, oral risedronate, and i.v. zoledronic acid have all demonstrated promise in preventing CTIBL in patients receiving hormonal therapy for breast cancer. In breast cancer and myeloma, bisphosphonates have now become part of standard therapy to treat and prevent skeletal-related events and, until recently, treatment was largely with intravenous pamidronate or oral clodronate. Zoledronic acid is the only bisphosphonate that has been compared directly with pamidronate, and it was shown by multiple event analysis to be significantly more effective at reducing the risk of a skeletal-related event (Coleman 2004). In patients with prostate cancer, clodronate, etidronate and pamidronate have demonstrated transient palliation of bone pain.

More recently, several large, long-term clinical trials have provided conflicting evidence of the clinical anti-tumour effects of oral clodronate. Diel *et al.* (1998) described a reduction in bone and visceral metastases, and an improvement in survival in patients with bone marrow-disseminated tumour cells. On the other hand, Saarto *et al.* (2001) reported increased incidence of bone and visceral metastases and a poorer survival in node-positive breast cancer patients after a median follow-up of 5 years.

However, zoledronic acid is the only bisphosphonate to demonstrate both significant and sustained pain reduction and a significantly lower incidence and longer time to onset of skeletal-related events compared with placebo (Lipton 2004). Zoledronic acid also has significant clinical benefit in patients with bone metastases from a variety of other solid tumours, including lung cancer and renal cell carcinoma (Coleman 2004, Lipton 2004).

1.6. Clinical use of MMPIs for cancer treatment

The first synthetic MMPIs were developed in the early 1980s. MMPs have been heralded as promising targets for cancer therapy on the basis of their massive up-regulation and activation together with tissue-destructive action on ECM and BM components in malignant tissues. Preclinical studies testing the efficacy of MMP suppression in tumour models were so compelling that synthetic MMPIs were rapidly developed and routed into human clinical trials. Nevertheless, the

results of these trials have been disappointing. First-generation MMPIs were hampered by poor bioavailability and were rapidly replaced by second-generation orally active drugs (Whittaker M *et al.* 1999). Marimastat was in phase III for the treatment of cancer, but it has been discontinued due to the lack of efficacy and musculoskeletal side-effects. All clinical trials with BAY-12-9566, CGS-27023A (MM-270, MMI-270) and prinomastat have been stopped as well (Peterson 2004).

Continued investment in MMPI development has been sustained despite a growing litany of failed clinical trials. At present the clinical use of neovastat and metastat is ongoing, and some novel MMPIs are in clinical development in cancer therapy (Peterson 2004). Metastat seems to be well tolerated, with photosensitivity and fatigue being the most frequently encountered side effects and it is being examined in the treatment of Kaposi's sarcoma (Peterson 2004). In phase I trials, metastat was found to induce disease stabilization in several patients who had a non-epithelial type of malignancy; metastat was also able to lower plasma MMP-2 levels, and it can suppress the production of both MMP-2 and MMP-9 when measured in plasma or skin biopsies in cancer patients (Rudek *et al.* 2001, Vihinen and Kähäri 2002). In these studies, phototoxicity was found to be dose-limiting, and in some patients treatment with metastat has been associated with reversible sideroblastic anaemia (Rudek *et al.* 2001). Metastat is currently in a phase I/II study in patients with progressive or recurrent high-grade anaplastic astrocytoma, anaplastic oligodendroglioma or glioblastoma multiforme (Vihinen and Kähäri 2002).

Neovastat is in phase III trials for the treatment of advanced non-small-cell lung cancer, metastatic renal-cell carcinoma and multiple myeloma (Falardeau *et al.* 2001). Furthermore, S-3304, rebimastat, CP-471358 and BMS-275291 are currently being investigated in phase I/II studies to evaluate the efficacy of these inhibitors. The broad-spectrum BMS-275291 has not shown musculoskeletal side-effects (Lockhart *et al.* 2003, Peterson 2004).

2. Aims of the Study

Proteolytic degradation of the ECM and BMs is essential in tumour progression. It allows cells to migrate beyond physical barriers, get to the blood stream and metastasize. Clinical trials with MMPIs have been disappointing because it is very likely that too excessive broad-spectrum blocking of MMPs lead to exclusion of the defensive or protective action of MMPs (Balbin *et al.* 2003, Owen *et al.* 2004).

Bisphosphonates are well-tolerated and frequently used drugs in bone diseases. The molecular mechanism of bisphosphonates, especially their effects on malignant cell migration and direct effects on MMPs, is not fully understood.

Furthermore, it is evident that gelatinases (MMP-2 and MMP-9) play a central and pivotal role in angiogenesis and affect the prognosis of cancer patients. When this research was initiated, no specific or selective gelatinase inhibitors or gelatinase-targeting compounds existed. The specific aims of this thesis were to study:

1. The effects of bisphosphonate (alendronate) on human fibrosarcoma, melanoma and endothelial cell invasion and random migration.
2. The direct effects of alendronate, clodronate, pamidronate and zoledronate on the catalytic activities of MMP-1, -2, -3, -9, -12, -13 and -20 and urokinase type plasminogen activator.
3. The effects of clodronate on the activation of proMMP-2, the expression of MT1-MMP mRNA and protein production by human MG-63 osteosarcoma cell line as well as on catalytic activity of pure human recombinant MT1-MMP.
4. To develop novel MMP-2 and MMP-9 inhibitors to target selectively human gelatinases expressing tumours.
5. To study the usefulness of these small, cyclic CTT1 and CTT2 peptides as anti-cancer agents *in vitro* and *in vivo* using human tongue carcinoma cells.

3. Materials and Methods

A summary of the methods used is described. Detailed methods are described in the original publications I-IV.

3.1. Cell lines and cell cultures (I, II, III, IV).

Human HT1080 fibrosarcoma, human C8161 melanoma, human umbilical vein endothelial (HUVEC) and its derivative Eahy926, human tongue squamous cell carcinoma HSC-3, human SKOV-3 ovarian carcinoma, KS6717 Kaposi's sarcoma-derived, human MDA-MB-435 breast carcinoma and human osteosarcoma MG-63 cell lines were maintained in DMEM or RPMI supplemented with 10% FCS, L-glutamine, penicillin-streptomycin andhypoxanthine/aminopterin/ thymidine additive with the Eahy926 cells and HEPES and endothelial cell growth supplement with HUVEC cells.

3.2. *In vitro* cell migration and invasion assays (I, III, IV).

Cell migration was studied using 8.0-mm-pore size and 6.5-mm-diameter Transwell inserts (Costar). Tumour cell invasion was studied using 6.4 mm-diameter Boyden chambers precoated with Matrigel (BD Biosciences). The layer of Matrigel matrix serves as reconstituted BM *in vitro*. The cells with or without various concentrations of bisphosphonates or peptides were allowed to migrate in the presence of 10% FCS containing culture medium. The cells that migrated to the underside of the membrane were stained with toluidin blue and quantitated by scanning with Bio-Rad scanner or counted under light microscope.

3.3. Cell viability, adhesion and proliferation assays (I, III, IV).

To assess the effect of different concentrations of bisphosphonates and peptides on cell viability, cells were plated in microtiter wells and after culturing for 20 hours, viability was determined with the MTT reagent according to the instructions of the manufacturer (Sigma). For cell adhesion studies microtiter wells were coated with fibronectin and blocked with BSA. Cells were added together with various concentrations of bisphosphonates or peptides and cultured for 1 h in a serum-free medium. After washing twice with PBS the bound cells were determined with the MTT reagent as above. To assess the effects of CTT1 and CTT2 peptides on cell proliferation, cells were plated in microtiter wells culturing for 24 h. The proliferation was determined with the Cell Proliferation ELISA, BrdU kit (Roche), where BrdU incorporation to newly synthesized DNA is measured in proliferating cells.

3.4. Measurement of mRNA expression (II, IV).

The total cellular protein and RNA were isolated by the Trizol® kit (Gibco) or Rneasy Mini Kit and QIAshredder (Qiagen) after culturing cells with various concentration of peptides in serum-free media. Ribonuclease protection assay (RPA) was carried out using RPA III™ Ribonuclease Protection Assay Kit (Ambion Inc.). Labelled MMP-9 RNA probe was hybridized with total RNA according to the manufacturer's instructions. The protected RNA fragment was run on 5% denaturing polyacrylamide gel and visualized on an X-ray film. A 28S probe was used as internal control.

For Northern blot analysis equal amounts of the RNA samples were fractionated on 0.7% agarose, 18% formaldehyde gels. The gels were stained with ethidium bromide for visualization of the rRNAs, and the RNA was transferred onto a filter after being photographed. Hybridization was carried out with 32P-labelled MMP-2 (2733 bp fragment) or MT-MMP1 (420-bp fragment) cDNA clones as recommended for Zeta-Probe (Sigma). The filters were washed and exposed to Kodak X-Omat film for 18 to 48 h with an intensifying screen at -70°C. The autoradiographs and photographs were analysed by quantitative densitometry using PhosphorImage.

3.5. Phage display and peptide synthesis (III).

Phage display peptide libraries CX_5C, CX_6C, CX_7C, CX_9 were prepared as described earlier (Koivunen *et al.* 1995). For selection of MMP-9-binding phage APMA-activated human neutrophil MMP-9 (100 µg/ml) was coated on microtiter wells at 4°C. The wells were then saturated with 5% BSA. In the first panning, the wells were incubated overnight at 4°C in 5 mM TBS buffer (Tris-HCL/0.1 M NaCl, pH 7.5) containing 1% BSA. After extensive washing, the bound phages were eluted with a low pH buffer. In subsequent pannings, the amplified phages were allowed to bind for 1 h at 22°C. Randomly selected clones were amplified over night and sequenced using Sequenase 2.0 kit (Amersham).

3.6. *In situ* zymography (IV).

Untreated frozen tissue sections were thawed and warmed to room temperature. Sections were covered with in situ zymography (ISZ)-buffer (50 mM Tris-HCl, pH 7.4; 1 mM CaCl2) alone, with 100 µM CTT2, CTT1 and C1 peptides, all dilutions in ISZ-buffer or 10 mM 1,10-phenanthroline. After 30 min incubation the solution was discarded and samples were covered with ISZ-mix consisting of DQ™ Gelatin (Molecular Probes) mixed 1:2 with 1% low melting point-agarose (Sigma) and 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) at 1 µg/ml was included to stain nuclei. The same inhibitors as used in the pre-incubation were added at indicated concentrations to the substrate solution. Samples were covered with equal amounts of ISZ-Mix with or without inhibitors and allowed to gel at room temperature for 30 min. Thereafter, samples were incubated at +37°C for 12-48 h. Green fluorescence was regarded as gelatinolytic activity in the otherwise uniformly dark quenched gelatin layer (Pirilä *et al.* 2001).

3.7. Mouse experiments (III, IV).

The animal experiments were approved by the Committee for Animal Experiments of Helsinki University (IV) and the Animal Care and Use Committee of the Burnham Institute (La Jolla, CA) (III). Human tumour xenografts were established in mice by administering 10^6-10^7 tumour cells per mouse in a 200-µl volume of serum-free DMEM. KS1767 and MDA-MB-435 cells were injected into the fat mammary pad of athymic mice, SKOV-3 cells into the peritoneal cavity of SCID/SCID mice (n=5) and HSC-3 cells subcutaneously into the right and left back of athymic mice (n=7 male and 9 female) (Harlan-Sprague-Dawley). To study the effect of CTT1 on tumour implantation, CTT1 was premixed with the tumour cells *ex vivo*, prior to the cell implantation. Alternatively, CTT1, CTT2 or control peptides were administered to established tumours either as a systemic (i.p. or i.v.) or as a local (s.c.) treatment. Tumour volumes were calculated and mouse survival was followed.

3.8. Colorimetric assay for MMPs using modified pro-urokinase as substrate (I, II).

The autoactivated and APMA (1 mM)-pretreated human MMPs were incubated with bisphosphonates at concentrations indicated in the text, and assayed for MMP activities according to Verheijen *et al.* (1997). The high and low Ca^{2+} concentrations were 5 and 1 mM $CaCl_2$, respectively, in 50 mM Tris-HCl, 150 mM NaCl, 1 µM $ZnCl_2$, 0.01% Brij, pH 7.8 (TNC buffer). The activities were expressed as relative units. uPA activity was assayed with chromogenic S2444 substrate (Chromogenix).

3.9. Measurement of type I collagenase activity (IV).

Collagenase activity was determined by incubating human recombinant collagenase-1 or MMP-1 (Calbiochem), collagenase-2 or MMP-8 (Chemicon International) and collagenase-3 or MMP-13 (Invitek) with 1.5 µM soluble native human skin type I collagen for 12 h at 22°C. MMP-1, -8 and -13 were pretreated with 100 µg/ml CTT2 in buffer at 37°C for 60 min with 1 mM APMA. The characteristic ¾- and ¼-cleavage products of type I collagen resulting from collagenase action were separated and analysed by 8% SDS-PAGE and quantitated by densitometry (Hanemaaijer *et al.* 1997).

3.10. Western blot analysis (II).

Western blot analysis for MMP-2 and MMP-13 was performed from cell culture media and for MT1-MMP from MG-63 osteosarcoma cell lysates. The samples were adjusted to contain equal amounts of protein for each experiment and samples were separated on 8 or 10% SDS-PAGE gels and electrophoretically transferred to a nitrocellulose membrane. Nonspecific binding was eliminated using PBS supplemented with 5% non-fat dry milk for 90 min at 37°C. The membrane was incubated with monoclonal antibodies against MMP-2, MMP-13, or with

polyclonal antibody against MT1-MMP (Calbiochem; dilution 1:500 for each antibody) for 10 h. The membranes were incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody for monoclonal antibodies and goat anti-rabbit IgG antibody for polyclonal antibody for 1 h. Thereafter the immunoblots were developed by addition of nitro blue tetrazolium (NTB), and 5-bromo-chloro-3-indolyl-phosphate (BCIP) diluted to N-N-dimethylformide in 100 mM Tris-HCL, 5 mM MgCl2, 100 mM CaCl2; pH 9.5. All incubations were performed at 22°C.

3.11. Gelatin zymography (I, II, III, IV).

The enzymatic activity and molecular weight of electrophoretically separated forms of gelatinolytic enzyme were determined from the cell culture media of tumour cells using gelatin zymography. Samples were incubated with Laemmli´s sample buffer after which the samples were separated in polyacrylamide (8 or 10% SDS) gel containing 1 mg/ml gelatin. The enzymes were renatured by series of buffers and incubated overnight at 37°C in buffer containing Ca^{2+} and Zn^{2+}. The reaction was stopped with Coomassie Brilliant Blue R250 staining, followed by destaining. The zymograms were quantified by densitometer scanning of the photos.

3.12. Enzyme inhibition assays (I, II, III, IV).

To determine the inhibitory effects of the synthetic peptides and bisphosphonates, purified MMPs were preincubated for 60 min with peptides and bisphosphonates at concentrations indicated in the text. The substrates, a 21-kDa β-casein (52 mM) or [^{125}I]-gelatin, and samples described above or cell culture media were incubated for 1 or 2 h at 37°C. Degradation of 21 kDa β-casein was analysed by SDS gel electroforesis. The degradation of [^{125}I]-gelatin was determined by counting radioactivity in the supernatant after precipitation of undegraded gelatin with 20% trichloroacetic acid. The radioactivity in the supernatant reflected gelatinase activity.

3.13. Immunohistochemistry (IV).

For the identification the carcinoma and endothelial cells, the frozen tissue sections were stained with CK-PAN and FVIII, respectively. Vectastain rabbit/mouse ABC Elite kits (Vector Labs) were used according to the protocol. The tumour tissue sections were fixed in acetone, incubated with 0.6% H_2O_2 in methanol for 30 min, and blocked with normal goat/horse serum (1:50) for 20 min. Sections were incubated overnight at +4°C with polyclonal FVIII antibody (dilution 1:5000, DAKO) and monoclonal CK-PAN antibody (1:700, DAKO). The protein-antibody complex was detected by incubating with biotinylated anti-rabbit/-mouse immunoglobulin G for 30 min and with avidin-biotin complex for 30 min. The slides were stained with 3-amino-9-ethylcarbazole (AEC) and counterstained with Mayer's haematoxylin. The microvessels were counted in four chosen x 400 fields in the highest vessel density areas. Serial sections of the slides were also stained with the van Gieson staining method.

3.14. Immunomagnetic isolation analysis and identification for HSC-3 cells in PBMC suspension (unpublished).

Peripheral blood mononuclear cells (PBMCs) were separated from buffy coat preparation of human blood by the modified dextran-Ficoll method. Dilution series of HSC-3 cells were prepared to determine the sensitivity of the detection system. The samples were resuspended HSC-3 cells at concentrations of 0, 10, 100 and 1000 cells in 2 x 10^7 PBMNCs in 1 ml of PBS/20% FCS. CELLection™ Epithelial Enrich Kit (Dynal, Oslo, Norway) contains superparamagnetic Dynabeads (4.5 µm diameter) coated to an antibody via a DNA linker. The antibody coated onto the CELLection™Dynabeads is an anti-EpCAM (epithelial cell adhesion molecule) mouse IgG1 monoclonal (Ber-EP4) specific for two (34 and 39 kDa) glycopolypeptide membrane antigens. Dynabeads with anti-mouse antibody only were used as a negative control. The different concentrations of HSC-3 cells with 2x10^7 PBMNCs were incubated with 2x10^7 immunobeads for 30 min at 4°C. Following incubation the cell suspension was placed against the magnet (Dynal MPC®) for 2 min to recover the carcinoma cells surrounded by the beads as rosettes. The supernatant containing the unbound cells was discarded. The test tubes were washed four times in 1000 µl RPMI/1% FCS. Cells were released using 200 units Dnase by incubating for 15 min at room temperature. The test tube was placed against the magnet for 3 min, and the supernatant containing the released cells was removed and transferred to a new test tube. 200 ml RPMI /1% FCS was added to resuspend the beads attached to the tube wall and after the magnet treatment, the supernatant was transferred to the same new test tubes. Cytotech slides were prepared from the released cells, air-dried overnight and stored at -80°C prior to immunocytochemical stainings.

The cytotech slides were stained with an EPIMET* epithelial cell detection kit (Baxter, Munich, Germany) according to the manufacturer's instructions (unpublished data). Briefly, cells were permeabilized, fixed and incubated with the conjugate of the murine monoclonal antibody A45-B/B3. The Fab fragments were conjugated to alkaline phosphatase (AP). Subsequently, an insoluble red reaction product (New Fuchsin) was developed at the binding site of the Fab-AP conjugate. The cells were counterstained with Mayer's haematoxylin to evaluate nuclear morphology. The slides were evaluated by light microscopy. Negative control staining experiments were performed with irrelevant (anti-FITC) isotopic antibodies.

To evaluate the expression of MMP-9 immunoreactive protein by HSC-3 cells the stainings were performed with polyclonal MMP-9 antibody (1:1000, Neomarkers, Fremont, CA, USA), using the VECTASTAIN®, Elite ABC Kit (Abbott, Chicago, IL, USA) as described above. Negative controls were stained with phosphate-buffered saline (PBS) and normal rabbit IgG instead of the primary antibody.

3.15. Immunofluorescence (II).

MT1-MMP expression in MG-63 cell monolayers was detected by using an indirect immunohistochemical staining (II). After the cells were grown at indicated concentrations of clodronate with serum-free medium, the cell layers were incubated with primary monoclonal MT1-MMP antibody (Oncogene Research Products™, Darmstadt, Germany), diluted at 1:80 in 1% BSA in PBS with 150 mM NaCl and incubated overnight in a humid chamber at 4°C. After washing the samples were incubated with fluorescein-conjugated rabbit anti-mouse immunoglobulins (1:60) (DAKO, Glostrup, Denmark) for 30 min at room temperature. The slides were examined by fluorescent microscopy.

3.16. Statistical analysis (I, II, III, IV).

Data are expressed as means ± standard deviation and the significance of differences between group means was determined by Student's t test. Data were considered as significant when at least $p=0.05$ was reached. Statistical differences in the Kaplan-Meier survival were determined by Log-Rank and Wilcoxon tests.

4. Results

4.1. Inhibition of human MMPs and uPA by bisphosphonates (I, II).

Pamidronate and zoledronate inhibited dose-dependently human macrophage metalloelastase (MMP-12) and enamelysin (MMP-20). Alendronate, pamidronate and zoledronate at a concentration range of 20-1000 µM inhibited the degradation of the 21-kDa β-casein band by human recombinant MMP-8, -3 and -13 in a dose-dependent manner. Alendronate and clodronate dose-dependently inhibited the activities of purified human MMP-1, -2, -3, -8, -9 and -13, but did not inhibit the serine proteinase uPA. The ability of 400 µM alendronate and clodronate to inhibit MMP-1, -2, -3, -8, -9 and -13 was reduced in the presence of high (5 mM) Ca^{2+} concentration in the assay buffer. In the presence of low or physiological (1 mM) Ca^{2+} concentration in the assay buffer the IC_{50} for MMP inhibition was 40-70 µM. Corresponding results were observed using ß-casein and radioactive gelatin-degradation assays. When 50 µM alendronate, pamidronate or zoledronate were added together with substrate to enzyme reactions, a clear inhibition was observed after 20-40 min.

4.2. The effects of alendronate on cell invasion, migration, viability and adhesion (I).

Alendronate at 50, 100 and 500 µM concentrations inhibited significantly, efficiently and dose-dependently the random migration and the invasion of HT1080 fibrosarcoma cells and C8161 melanoma cells through type IV collagen-coated Matrigel cell culture inserts (Table 2). Futhermore, alendronate reduced dose-dependently and significantly the migration of human endothelial cell lines (Eahy926 and HUVEC). As a positive control for the cell invasion and random migration assays we used CTT1 (500 µM), which significantly reduced the Matrigel invasion and random migration of the HT1080 fibrosarcoma, C8161 melanoma and endothelial cell lines (Table 2). Alendronate did not decrease the growth of C8161 melanoma cells at any of the concentrations studied. At the 500 µM concentration, alendronate partially decreased the growth of HT1080 fibrosarcoma cells during a 24-h culture, but did not affect the cells at the 50 or 100 µM concentrations, which are the concentrations that significantly inhibited cell invasion and random migration. Furthermore, alendronate significantly promoted the adherence of both HT1080 fibrosarcoma and C8161 melanoma cell lines to fibronectin and Matrigel substrata when compared to untreated cell lines. The CTT1 peptide did not affect cell adhesion for fibronectin, was not toxic to cells and did not affect cell viability.

Table 2. Effect of alendronate on cell migration and invasion. The respective levels of dose-dependent inhibition (%) of HT1080 cell and C8161 cell migration and invasion compared to buffer alone.
ND= not determined, n=3, $p \leq 0.05$

	Migration			Invasion		
Cell lines	**Alendronate**			**Alendronate**		
	50	**100**	**500µM**	**50**	**100**	**500µM**
HT 1080	29%	53%	87%	72%	84%	98%
C 8161	31%	41%	65%	62%	77%	86%
HUVEC	23%	34%	95%	ND	ND	ND
Eahy926	58%	69%	92%	ND	ND	ND

4.3. Proteolytic activation of MMP-2 by PMA and the effects of clodronate on MMP-2 activation in human osteosarcoma cells (II).

Densitometric analysis of 72 kDa proMMP-2 activation induced by PMA showed that 10^{-7} M PMA treatment for 24 h resulted in the production and conversion of Mr 72 kDa latent proMMP-2 to Mr 62 kDa active MMP-2 species in the conditioned medium of human MG-63 osteosarcoma cells, thus evidencing clear MMP-2 activation. Human MG-63 osteosarcoma cells were then exposed to different concentrations of clodronate and the resulting culture supernatants were evaluated accordingly by gelatin zymography. In PMA-induced MG-63 cell culture media the percentage of the activated form of MMP-2 in respect to total MMP-2 amount was 11.8 (SD±0.25), and 0.1 mM clodronate decreased this percentage to 10.9 (SD±0.78, 1.74, p=0.5; n = 6). A significant decrease to 4.3% (SD±0.78, p<0.01) and to 0% (SD±0, p<0.01) was observed with 1.0 mM and 2.5 mM clodronate concentrations, respectively.

4.4. MMP-2 and MT1-MMP mRNA expression (II).

Total RNA was extracted from MG-63 cells, and the steady-state levels of MT1-MMP and MMP-2 transcripts were compared by Northern analysis. Relative hybridization signal numbers were calculated by ascribing an arbitrary value of 1 to the signal seen on the Northern blot of MG-63 cells without clodronate treatment after dividing the scanning units of mRNA levels per corresponding ribosomal RNA units determined using a PhosphorImager. The relative MT1-MMP mRNA expression levels were 1, 0.52, 0.36, and 0.25 for 0 mM, 0.1 mM, 1.0 mM and 2.5 mM of clodronate, respectively, and the relative levels of MMP-2 mRNA expression were 1, 0.76, 0.68, and 0.50 for 0 mM, 0.1 mM, 1.0 mM, and 2.5 mM of clodronate, respectively.

4.5. Production of MMP-2, MMP-13, and MT1-MMP in human osteosarcoma cells and gelatinolytic activity of MT1-MMP in conditioned human cell culture medium (II).

In the Western immunoblot analysis the protein production was evaluated using antibodies against MMP-2, MMP-13 and MT1-MMP. Both MMP-2 and MMP-13 were detected from the cell culture media of MG-63 cells before and after clodronate treatment. The antibody for MMP-13 did not detect MMP-13 protein in human MG-63 osteosarcoma cells. Using specific polyclonal antibody for MT1-MMP was seen the presence of MT1-MMP in cell lysates of sizes 56/62 kDa. Additional 56/62 gelatinolytic bands could also be detected in zymograms.

4.6. Immunofluorescent staining of MT1-MMP in osteosarcoma cell monolayers (II).

Immunofluorescent cytochemistry of the MG-63 cell line for MT1-MMP was performed using mouse anti-human MT1-MMP monoclonal antibody, followed by fluorescein-conjugated rabbit anti-mouse IgG. We found that MT1-MMP was distributed at the cell surface in MG-63 cells treated with PMA. Incubation with 0, 0.1 mM and 1.0 mM clodronate dose-dependently decreased the steady-state pericellular expression of MT1-MMP immunoreactivity.

4.7. Clodronate inhibits the activity of catalytic domain of human recombinant MT1-MMP (II).

Clodronate in the concentration range of 0 to 2.0 mM inhibited the activity of the catalytic domain of human recombinant MT1-MMP. The IC_{50} was found to be 40 to 70 µM. The ability of 400 µM clodronate to inhibit MT1-MMP was reduced in the presence of high (5mM) Ca^{2+} concentration in the assay buffer when compared to physiological (1 mM) Ca^{2+} concentration.

4.8. Selection and early characterization of MMP-9-binding peptide (III)

The CTTHWGFTLC (abbreviated CTT1) peptide is one of three (CRRHWGFEFC, CTTHWGFTLC, CSLHWGFWWC) ten-amino-acid sequences found by panning of CX9 library against MMP-9. All the peptides found have a conserved HWGF sequence and cysteines at both ends forming a circular peptide when oxidized. The CTT1 and CRRHWGFEFC inhibited gelatine degradation by MMP-9 and MMP-2 at low micromolar concentrations. CTT1 peptide was chosen after a more detailed investigation because of its more favourable solubility. The importance of cyclizations was tested by creating an analogue of the CTT1 peptide that had serines at the ends instead of cysteins. This linear STTHWGFTLS peptide lacked almost completely the inhibition

activity of CTT1. CTT1 did not inhibit other members of the MMP family or other classes of proteinases.

4.9. Inhibition of cell migration by CTT1 (III).

CTT1 inhibited migration of HT1080 fibrosarcoma, C8161 melanoma, SKOV-3 ovarian carcinoma, KS1767 Kaposi's sarcoma, Eahy 926 and HUVEC endothelial cells in a dose-dependent manner (20-500 µM). The maximal inhibition (80%) was seen at a peptide concentration of 500 µM. Matrigel invasion of HT1080 and C8161 cells was also suppressed by the CTT1. Maximal inhibition was 90% at 500 µM, the highest concentration studied. None of the control peptides affected migration or invasion. CTT1 was not toxic to the cells, as cell viability did not decrease even when the cells were cultured as long as two days in the presence of the peptide. Initial attachment and spreading of the cells on fibronectin, collagen type IV, or Matrigel substrata was not affected by the peptide.

4.10. Prevention of tumourigenesis and inhibition of tumour growth in mice (III).

MDA-MB-435 breast carcinoma cells were preincubated with CTT1 (100 µg) or vehicle alone (DMEM) and injected subcutaneously into nude mice. CTT1 delayed formation of tumours (follow-up time was 5 weeks), and the tumours that arose were significantly smaller than those in the control group.

MDA-MB-435-derived breast carcinomas were established in the mammary fat pad nude mice. CTT1 (200 µg per mouse) or a control peptide was injected into the subcutaneous tissue adjacent to the tumour three times a week for three weeks. The volumes of tumours treated with CTT1 remained stable, whereas the tumours treated with the control peptide progressed, reaching up to five times the initial volume at the end of the experiment.

Mice bearing established KS1767-derived Kaposi's sarcomas were injected intraperitoneally with CTT1 (100 µg/mouse) or control peptide three times a week for four weeks. KS1767-derived Kaposi's sarcomas grew more slowly in mice treated with intraperitoneal injections of CTT1 than in the control mice treated with the vehicle alone. In addition, establishment and growth of SKOV-3-derived ovarian carcinomas was also inhibited when CTT1 (200 µg) was coinjected with the cells into the peritoneal cavity of severe combined immune-deficient (SCID) mice. CTT1 increased the survival of the mice bearing SKOV-3 tumours as compared with the control group, which received a control cyclic peptide. Similarly, the survival of SCID mice bearing i.p. SKOV-3 tumours also improved upon treatment with CTT1. In two independent experiments, four out of six and three out of five of the peptide-treated mice were alive at the time when all the control mice had died from their tumour burden.

4.11. Targeting of CTT1 phage into tumours (III).

CTT1 sequence-containing phages were inserted intravenously to mice bearing size-matched KS1767-derived Kaposi's sarcoma tumours (approximately

1,000 mm^3) and recovered after perfusion. Tumour homing ability of the phage was elevated ten-fold compared to phage having random peptide sequence. The tumour- to brain-ratio was also approximately ten-fold. Moreover, the homing of the CTT1 phage into the tumours was specific, as it could be substantially inhibited upon coinjection of the cognate peptide (CTT1). Coinjection of an unrelated cyclic peptide (CARAC) had no appreciable effect.

4.12. The effect of CTT1 and CTT2 peptides on pro-MMP-9 conversion and gelatinolytic activity of the cultured HSC-3 tongue carcinoma cells (IV).

The serum-free HSC-3 conditioned medium revealed that hydrophobic CTT1 and it's newly developed derivative hydrophilic GRENYHGCTTHWGFTLC (abbreviated CTT2) peptides at concentrations from 20 to 100 µg/ml markedly reduced the 92 kDa pro-MMP-9 conversion to 77-82 kDa active forms in gelatin zymography. CTT2 did not affect the level of conversion of MMP-2. We also studied the effect of the peptides on the catalytic activities of secreted gelatinases using the iodinated gelatin degradation assay. CTT1 and CTT2, at concentrations of 20, 50 and 100 µg/ml, decreased the activity of conditioned media gelatinase to 58% and 75% (SD: ± 1.4% and ± 4.2%), 42% and 54% (SD: ± 8.5% and ± 11.3%) and 29% and 24% (SD: ± 5.7% and ± 24.0%), respectively. Furthermore, the RPA analysis of various concentrations (20, 50 and 100 µg/ml) of CTT1 and CTT2 peptides showed no effect on the MMP-9 mRNA expression levels of HSC-3 cells.

4.13. The effects of CTT1 and CTT2 peptides on HSC-3 cell fibronectin adhesion, proliferation, viability, migration and invasion *in vitro* (IV).

At a concentration of 100 µg/ml, CTT2 partially decreased the viability of HSC-3 cells during a 24-h culture period, but did not affect the cells at the 50 or 20 µg/ml concentrations. There was no effect on cell viability by CTT1 with the concentrations used. HSC-3 cell proliferation and cell adhesion on fibronectin were not affected by CTT1 or CTT2 at a 100 µg/ml concentration.

The CTT1 and CTT2 peptides at 20, 50 and 100 µg/ml concentrations efficiently and dose-dependently inhibited the random migration and the *in vitro* invasion of human tongue HSC-3 cells through Matrigel cell culture inserts (see Table 3). The control C1 peptide had no effect on cell migration and invasion at a concentration of 100 µg/ml. CTT2 was significantly more effective than CTT1 in both assays ($P \leq 0.024$).

Table 3. Inhibition (%) of HSC-3 cell migration and invasion with CTT1 and CTT2.
n = 3, p ≤ 0.05

HSC-3 cells	Migration			Invasion		
	20	50	100µg/ml	20	50	100µg/ml
CTT1	38%	51%	66%	25%	34%	54%
CTT2	43%	68%	73%	28%	59%	69%

4.14. Inhibition of tumour growth and angiogenesis by CTT2 in mice (IV).

The effect of the CTT2 peptide on tumour growth *in vivo* was analysed by injecting HSC-3 tongue carcinoma cells subcutaneously into athymic mice, followed by beginning the peptide injections three days after. CTT2 (400 µg), the control peptide (C2 peptide, 400 µg) or PBS were injected intravenously once a day for five days. The growth of the tumours treated with CTT2 was slow, whereas the tumours treated with C2 or PBS gradually progressed, reaching up to five times the initial volume at the end of the experiment. Furthermore, the tumours treated with CTT2 faded and tumour size was reduced. CTT2-injections significantly ($P \leq 0.012$) increased the survival of the mice. After one month all the mice treated with C2 or PBS had succumbed to the disease, whereas one-third of the CTT2-injected mice were alive after two and a half months. There was no difference in tumour growth and survival between the genders. We also examined the effect of CTT2 on the angiogenesis in female and male mice bearing human tongue SCC tumours by immunodetection of cells expressing the endothelial cell marker FVIII. Immunohistochemical analysis of the tumours revealed that the blood vessel density was significantly lower ($P \leq 0.043$) in the CTT2 peptide group than in the C2 and PBS groups. All the tumour tissue sections were stained with CK-PAN to evidence that the tumours had originated from the carcinoma cells. Furthermore, we demonstrated the cell-matrix interactions by van Gieson histological staining. In the C2- and PBS-treated groups the tumour cells were large, disordered, loosely orientated and surrounded by thin interrupted collagen fibres, whereas in the CTT2-treated tumours the carcinoma cells remained mainly small and faded, and the tumours were surrounded by a continuous collagenous and fibrous capsules.

4.15. Inhibition of human tongue SCC tissue gelatinolytic activity by CTT1 and CTT2 as assessed by in situ zymography (IV).

In human native tongue SCC tissue sections gelatinolytic activity could be clearly detected in the carcinoma cells and extracellular matrix. Gelatinolytic activity was almost completely abolished with 100 µg/ml of CTT1 or CTT2

peptide, whereas the control peptide (C1) did not affect the gelatinolytic activity of these carcinomas.

4.16. Recovering HSC-3 cells with immunomagnetic method and isolation and production of MMP-9 by HSC-3 cells (unpublished data).

In the immunomagnetic method the mean recovery of the seeded HSC-3 cells was close to 46% (Table 4). Even in the experiments seeding only 10 cells the recovery always exceeded zero, being at least 4 captured carcinoma cells (Table 4). Each captured HSC-3 cell stained positively with the polyclonal MMP-9 antibody (not shown).

Table 4. Results of recovery experiments using the immunomagnetic method

Approximal number of seeded HSC-3 cells in $2x10^7$ MNCs				
	0	10	100	1000
Range (n=8)	0	4-30	23-116	120-790
Mean recovery	0	17	91	401
Standard deviation	0	9,23	36,07	268,14

Each experiment was performed 8 times. The values in the boxes show the range of cell numbers recovered, the mean recovery and the standard deviation for each number of seeded HSC-3 cells.

5. Discussion

Since the discovery of the first archetypal MMP (MMP-1) over four decades ago by Gross and Lapierre (1962), most major pharmaceutical companies have had MMP inhibitor programmes for a variety of tissue destructive diseases. During decades of intensive research, tens of thousands of compounds have been synthesized and screened as potential MMP inhibitors, and large amounts of money have been spent on *in vitro* and *in vivo* studies as well as on clinical trials. So far, to date, Periostat (doxycycline hydrate, a tetracycline analogue) is the only MMPI licensed in the United States, and the application is for periodontal disease (Wynn 1999). In the targeting of MMPs for cancer therapy, it is important to recognize that these proteases are mainly expressed by surrounding stromal fibroblasts, vascular cells, and by the inflammatory cells infiltrating tumours, rather than by the cancer cells themselves (Bissell and Radisky 2001).

5.1. The lack of efficacy of MMPIs in cancer treatment

Why are MMPIs not effective in clinical trials in cancer treatment despite abundant proof-of-concept *in vitro* and *in vivo* studies? Most clinical trials of MMPIs have yielded disappointing results, perhaps due to inappropriate study design or tumour staging, or to lack of selectivity of broad-spectrum MMPIs used, and to old ways of thinking that MMPs only degrade ECM, without realizing that MMPs also influence various non-matrix substrates (Coussens *et al.* 2002, Overall and Lopez-Otin 2002, Peterson 2004). Firstly, MMPI clinical trials have typically targeted late-stage cancer patients while most preclinical studies manipulated MMP activity prior to the induction of cancer. Animal experiments show that MMPs should be targeted early in the cancer progression. Batimastat reduces endpoint tumour burden in an animal model of progressive pancreatic carcinoma if given at the early hyperplastic stage, but the effects are limited if treatment begins at later stages (Bergers *et al.* 1999). Secondly, classical animal models of subcutaneous or intravenous injection of human tumour cells into immunodeficient mice are insufficient to evaluate the activity of cytostatic agents such as MMPIs because they do not adequately recapitulate host-tumour interactions (Peterson 2004). Malignant cells are genetically highly unstable so therapies targeting these cells can result in modifications resulting in resistance to cancer therapy. MMP activity in cancers usually originates from both tumour and stroma and importantly, stromal cells are much less likely to develop drug resistance (McCawley and Matrisian 2000). Thirdly, certain MMPs can have dual effects on cancer development (Andarawewa *et al.* 2003), or some functions of MMPs might help defy cancer (Balbin *et al.* 2003, Hamano *et al.* 2003, Pozzi *et al.* 2002). Furthermore, these MMPIs may also target other proteases, such as the ADAMTSs, which have the ability to slow down tumour growth through their antiangiogenic activity (Vazquez *et al.* 1999). Therefore it might be important to target better specific MMPs or specific functions of MMPs.

Fourthly, the dosage used in some trials could, however, also have been too low because of the side effects of MMPIs: the most commonly MMPIs cause musculoskeletal pain, which is evidently due to the inhibition of sheddases. Sheddases are responsible for release of membrane-bound proteins, including TNF-α.

There is no reliable biomarker in phase I studies, which could serve as a guide for dose-selection and administration schedule for phase II. As known for CMTs, application of the drug during activation of MMPs *in vitro* drops IC50s dramatically in comparison to adding the drug after activation (Golub *et al.* 1998). Biomarkers may not only be valuable in helping us understand how to derive optimal biological dose and dosing schedule, but may also be used in *in vitro* and *in vivo* studies to define target identification. For example, MMP-11 and -14 expression levels are negative prognostic indicators in small-cell lung cancer, and this type of tumour has undetectable expression of MMP-2 (Michael *et al.* 1999). Tanomastat, which targets MMP-2 but lacks MMP-11 inhibitory activity, may not be the best choice for this type of cancer. In fact, tanomastat treatment accelerated mortality in patients with pancreatic and small-cell lung cancer, which lead to the withdrawal of this drug for both arthritis and cancer treatment (Peterson 2004). Analysis of the serum or plasma levels of MMPs has been largely uninformative (Hidalgo and Eckhardt 2001).

MMPIs are cytostatic rather than cytotoxic, so conventional measures of efficacy such as reduction in tumour size, could not be used to monitor drug activity. Thus they do not directly cause shrinkage of established tumours. Phase I trials were often followed immediately by phaseII/III combination trials without the benefit of efficacy information from smaller studies (Peterson 2004).

5.2. Use of MMP inhibiting bisphosphonates in cancer treatment

Evidence has been accumulated from *in vitro* and *in vivo* studies that bisphosphonates can directly affect tumour cells. They can induce tumour cell death, for example by inducing cell apoptosis or inhibit their growth and spread. They may also indirectly reduce cancer growth by cutting-off their 'survival factors' such as TGF-β and IGF-I, which are often released from the resorbing bone. Based on these effects they may represent a new and alternative class of drugs with antitumour power (Green 2003).

Bisphosphonates directly inhibit the activity of MMPs, which is obligatory for successful down-regulation of tumour cell invasion. The mechanisms of MMP inhibition evidently involve the ability of bisphosphonates to act as cation chelators, as demonstrated in our studies showing that pamidronate and zoledronate inhibited dose-dependently MMP-12 and MMP-20. Alendronate, pamidronate and zoledronate inhibited MMP-8, -3 and -13 and alendronate and clodronate inhibited MMP-1, -2, -3, -8, -9, -13 and 14 (I, II). Other studies confirm and further extend our results; e.g. tiludronate inhibits MMP-1 and MMP-3, and clodronate inhibits MMP-8 and MMP-1 (Nakaya 2000, Teronen *et al.* 1997a, b, Boissier *et al.* 2000). Alendronate inhibited the invasion of

fibrosarcoma and melanoma cells (I) similarly to breast and prostate cancer cells through artificial membrane *in vitro* as published by Boissier *et al.* 2000. This is thought to be due, at least in part, to the inhibitory effect of bisphosphonates on MMPs. However, the specific inhibition of distinct MMPs seems to differ with various bisphosphonates. Certain bisphosphonates also exhibit effects on MMPs production by cancer cells. Valleala *et al.* (2003) have shown that monocytes/macrophages MMP-9 mRNA levels remain relatively stable in the presence of clodronate, but in contrast, pamidronate increases mRNA levels even up to 10-fold. However, clodronate dose-dependently down-regulated, but pamidronate up-regulated MMP-9 secretion at lower concentrations. In contrast, using higher concentrations of pamidronate MMP-9 secretion decreases (Valleala *et al.* 2003). Zoledronic acid causes a significant reduction in MMP-7 levels and an even greater proportionate increase in the levels of its inhibitor TIMP-2 in prostate cancer cells in bone marrow stroma co-cultures (Montague *et al.* 2004). On the other hand, Denoyelle *et al.* (2003) did not see an increase in MMP-2 and -9 levels at the 1 μM zoledronic acid concentrations in breast cancer cells. In our studies, clodronate inhibited PMA-induced proteolytic activation of proMMP-2 by human osteosarcoma cells (II). Furthermore, clodronate also downregulated expression of MT1-MMP mRNA and protein production in human osteosarcoma cells and inhibited MT1-MMP activity (II). Thus intracellular mechanism involves downregulation of induced MT1-MMP mRNA and protein expression (II). These findings may, at least in part, explain the antitumour activities of clodronate at molecular level. We did not see any effect of alendronate on fibrosarcoma and melanoma cell adhesion (I) similarly to Riebeling *et al.* (2002), who analysed clodronate in melanoma cell adhesion. However, there are studies demonstrating that bisphosphonates exert effects on cancer cell adhesion (Brown *et al.* 2004).

In our study, alendronate clearly inhibited the migration of human umbilical vein endothelial cells (I). Other studies confirm that bisphosphonates can inhibit angiogenesis, that is essential to tumour growth and metastases (Giraudo *et al.* 2004). Zoledronic acid suppressed MMP-9 expression in a mouse model involving human cervical cancer by infiltrating macrophages and inhibited metalloproteinase activity, reducing association of VEGF with its receptor on angiogenic endothelial cells (Giraudo *et al.* 2004). Zoledronic acid was found to be antiangiogenic, producing effects comparable to MMP-9 gene knockout mice in impairing angiogenic switching, progression of premalignant lesions and tumour growth. Zoledronic acid therapy increased neoplastic epithelial and endothelial cell apoptosis without affecting hyperproliferation, indicating that zoledronic acid was not antimitotic (Giraudo *et al.* 2004). Furthermore, zoledronate sensitizes endothelial cells to TNF-induced programmed cell death (Bezzi *et al.* 2003).

On the other hand, 0.0001-10 μM alendronate inhibits invasion of PC-3 prostate cancer cells by affecting the mevalonate pathway, but alendronate had no effects on the levels of MMP-2 or MMP-9 activity accumulated during the invasion assay (Virtanen *et al.* 2002).

The mechanism of MMP inhibition remains a matter of speculation, although

it is possible that it is linked to the primary inhibition of the mevalonate pathway and Ras inactivation. Inhibition of the Ras/Rho/MAPK pathway, linked through the mevalonate pathway, is known to inhibit MMP production in some tumour cell types (Shin *et al.* 2002), and inhibition of Ras *in vitro* will also block the production of MMPs (Futamura *et al.* 2001). These findings suggest that bisphosphonates may be useful agents for the prophylactic treatment of patients with cancers that are known to metastasize preferentially to bone.

However, doctors in the USA have during the past few years met a cluster of patients treated with bisphosphonates suffering from necrotic lesions in the jaw. The typical presentation is a non-healing tooth-extraction socket or exposed jawbone, and the clinical outcome was similar to osteoradionecrosis. The pathogenesis of jaw lesions may be related to potent osteoclastic inhibition, altered blood flow in bone, or localized antiangiogenesis by bisphosphonate medication (Ruggiero *et al.* 2004). Bagan *et al.* (2005) present a series of ten patients with osteonecrosis of the jaw that appeared following cancer chemotherapy without evidence of metastatic disease to the jaws. All the patients had received treatment for their malignant bone disease with bisphosphonates.

The fact that bisphosphonates have been used clinically for a number of years and are well known both pharmacologically and toxicologically suggests that they should be investigated in properly designed studies in patients with cancer for which inhibition of MMPs might be beneficial. Detailed knowledge of MMP inhibition spectrum of the bisphosphonates used and the identification of protease profiles of each patient's tumour, will enable us to develop targeted therapies with reduced toxicities. These clinical observations and preclinical evidence of antitumor activity of bisphosphonates provide a compelling basis to study further their antineoplastic efficacy in cancer patients. We need further research to fully elucidate the molecular mechanism involved and to determine the most effective dose and medication schedule of bisphosphonates to maximize their antitumour potential in the clinical setting, either alone or in combination with standard antineoplastic agents or other anti-MMP drugs (Llavaneras *et al.* 2001, 1999).

5.3. Novel approaches for MMP inhibition in cancer

Clinical trials have so far focused on patients with advanced-stage disease. Based on animal experiments, we would expect that the clinical efficacy might be improved either by using MMPIs in the treatment of early stages of the disease (in combination with conventional therapy), or as preoperative and postoperative treatment to prevent micrometastatic spread and recurrence of the disease. Thus the best therapeutic window for the MMPIs may be lost if the disease cannot be diagnosed early enough. Oral cancer, which is aggressive and has a high potential for invasiveness associated with high mortality, is often diagnosed in an advanced stage. The five-year survival rate of early-stage oral cancer is approximately 80%, while the survival drops to 19% for late-stage disease, and the rate of second primary tumour development in these patients has been reported to be 3-7% per year, higher than for any other malignancy (Murphy *et al.* 1995, Day and Blot 1992).

Endothelial cells, migrating tumour cells and inflammatory cells in the near proximity of tumours secrete gelatinases in a manner that corresponds to poor prognosis in cancer. For example, it is known that the expression of MMP-9 is a negative prognostic indicator for head and neck squamous cell carcinoma (Ruokolainen *et al.* 2004). These findings highlight the need for synthetic MMPIs that would selectively target specific MMPs. Our group created the cyclic, hydrophobic decapeptide (CTT1), which is the first gelatinase-selective peptide inhibitor (III). Thereafter we generated the hydrophilic, derivative of the peptide (CTT2) having a GRENYHG-tail (IV). In this study (III, IV) these peptides (CTT1 and CTT2) were found to inhibit the gelatinolytic activity of MMP-2 and MMP-9, but not that of other MMPs or serine proteinase (human neutrophil elastase, neutrophil cathepsin G and tumour-trypsin-2), cell migration and invasion of various tumour cells *in vitro*, as well as tumour progression *in vivo* in mouse models, indicating the importance of gelatinases (MMP-2 and MMP-9) in tumour invasion. However, the exact mechanism how CTT1 and CTT2 inhibit gelatinase activity is not completely known (III, IV), but the hydrophilic CTT2 was shown to be a slightly better inhibitor than hydrophobic CTT1 *in vitro* (IV). CTT1 exhibited strong tumour-homing ability in comparison to the normal tissues (III). This knowledge could guide the selection of a more appropriate tumour types for the clinical testing of CTT1 or CTT2, which target MMP-2/MMP-9 and have very little activity toward other MMPs (III, IV).

Both CTT1 and CTT2 peptides can be easily degraded *in vivo*, restricting their oral availability. The targeting capability of CTT1 was further demonstrated with liposomes coated with the CTT1 peptide. Hydrophobic CTT1 combined with adriamycin-containing liposomes enhanced up to 4-fold the killing of leukaemia and sarcoma cells compared to the control liposomes without the peptide. CTT1 has also been used for the delivery of anti-cancer drugs to tumours, based on its ability to home to tumours expressing MMP-2 and MMP-9 (Medina *et al.* 2001). CTT1 is thus not only a potential anti-cancer compound per se due to its selective gelatinase inhibitory activity, but it can also be used as a tool for more targeted delivery of other anti-cancer drugs as well as gene delivery (Medina *et al.* 2005). In this way, the development of more specific MMPIs may also indirectly have benefits in the treatment of cancer patients. In the future it would be important to analyse the effectiveness of hydrophilic CTT2-bearing liposomes containing a cytostatic drug to selectively target and kill the tumour cells and neo-vessels by targeting the site of MMP-9 action.

The evaluation of MMPIs in immune-deficient mice transplanted with human tumours is the major model system for drug development. This model allows rapid and quantifiable assessment of antitumour activity relative to mouse toxicity. However, these kind of preclinical studies do not accurately predict human efficacy. The organotypic gel culture system, which is generated by plating tumour cells on to a synthetic stroma composed of a collagen gel embedded with fibroblasts, allows carcinoma cells to grow in a 3D state. This model stimulates the tumour-stroma interaction and recapitulate more closely the *in vivo* situation (Nyström *et al.* 2005).

The identification of the specific proteases that must be targeted in cancer

should also be correlated with the design of synthetic 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, as well as to identify the *in vivo* substrates that individual MMPs can targ*et al*one or in co-operation with other proteolytic systems, the hydrolysis of which may strongly influence the behaviour of tumour cells. In addition, a better understanding of the regulatory mechanisms that control individual 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 (Medina *et al.* 2005), identification of surrogate markers to monitor MMP inhibition (Mäntylä *et al.* 2003, Emingil *et al.* 2004a, b), and design of appropriate combinations of MMPIs with cytotoxic and other anti-MMP drugs (Llavaneras *et al.* 1999, 2001), may finally lead to effective MMPI-based therapies for cancer (Overall and Lopez-Otin 2002).

One of the major questions is whether the MMPs in general are a clinically relevant target for the therapeutic intervention in cancer, and to what extent other tissue destructive diseases could be treated with the broad-spectrum MMPIs. Although over a hundred small-molecules targeting the catalytic site of the MMPs, have been synthesized, these broad-spectrum MMPI-compounds have limited specificity to individual MMPs and to date no significant success has been seen with these compounds in clinical trials. At present, sufficient or specific knowledge of the role of individual MMPs in cancer or other tissue-destructive diseases is lacking. Clearly, the function of MMPs is only now emerging. Hence, the decision as to which individual MMP should be targeted still remains an educated guess. Even if such knowledge were available, the conserved structural features of the MMPs indicate that it will be a considerable challenge to synthesize an active-site inhibitor with the selective specificity to a single MMP.

It remains to be seen whether the more selective active-site inhibitors, exosite inhibitors and inhibitors of protein-protein interactions such as those identified in this study (III, IV), appear to be any more successful as cancer therapeutics. A better understanding of both the destructive (Kähäri and Saarialho-Kere 1999, Sorsa *et al.* 2004) and the defensive (Balbin *et al.* 2003, Owen *et al.* 2004) roles of MMPs in cancer progression is certainly required in order to achieve the use of MMPI as cancer therapeutics (Overall and Lopez-Otin 2002). The recent finding that MMP-2 interacts with the chaperone protein Hsp90 in the extracellular space and that this interaction regulates tumour cell invasion is one indication that we do not yet understand the details of tumour cell migration and invasion (Eustace *et al.* 2004). Furthermore, blocking MMPs alone may not be sufficient to achieve an adequate clinical response. Hence, it is certainly worthwhile to consider the possibility of combination therapy with drugs affecting other functions of cancer cells. However, what has not been sufficiently appreciated to date is that a minimal (but not excessive) activities and levels of these MMPs have physiological, anti-tumour and anti-inflammatory properties by processing certain growth factors and anti-inflammatory cytokines (Overall and Lopez-Otin 2002, Owen *et al.* 2004). In this regard, complete inhibition

of MMPs is not desired, but instead reduction of pathologically excessive MMP activities and levels should preferably be targeted (Sorsa and Golub 2005).

Taken together, these findings emphasize 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 identify precisely 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). Nevertheless, bisphosphonates and the peptides identified in this study as MMP inhibitors/down-regulators (I, II, III, IV) will hopefully be useful in addressing in more detail the role of MMPs in physiological and pathological conditions and to aid in the development of pharmacologically relevant and active agents to combat cancer and other diseases associated with pathologically excessive MMP activity.

5.4. The use of immunomagnetic method in detecting circulating tumour cells

Detection of circulating tumour cells and their MMP-9 expression in cancer patients can be useful in determining prognosis and monitoring systemic therapies. We used an immunomagnetic method to isolate, identify and quantify human tongue carcinoma cells from the peripheral blood mononuclear cell suspension. Circulating epithelial tumour cells are extremely rare and are present at a frequency of 1-10 in 10^6 cells, and thus this method was found to be very sensitive and specifically capable of detecting as few as one tongue carcinoma cell in 2×10^7 in mononuclear cell suspension. Our results are comparable with the recent studies using immunomagnetic separation with various cancers (Werther *et al.* 2000, Eaton *et al.* 1997, Kruger *et al.* 2000, Nakamura *et al.* 2000, Wirtschafter *et al.* 2002). The detection of circulating tumor cells as a tumour marker may be used to monitor tumour progression and to evaluate progression-free and overall survival of prostate and breast cancer patients (Ady *et al.* 2004, Gaforio *et al.* 2003, Soria *et al.* 1999, Luke *et al.* 1998). However, Marth *et al.* 2001 reported that circulating tumour cells in the peripheral blood of patients with ovarian carcinoma did not predict prognosis. Furthermore, in a study analysing MMP-1, -2, -7 and -9 production in ten oral SCC patients by RT-PCR from laser-captured microdissection of cancer tissue and immunomagnetic separation of circulating cancer cells, only MMP-9 positive samples were detected (Ito *et al.* 2003). As we obtained promising results, we suggest that our study should be extended with long-term follow-up studies of cancer patients to investigate the primary tumour and circulating cancer cells and their MMP-9 expression in order to evaluate their prognostic value. The samples from cancer patients should be taken before surgical treatment; this helps to avoid detecting tumour cells which shed after surgical treatment from primary tumour. Therefore, we suggest that measurement of MMP-9 levels in primary tumours and micrometastases and the use of CTT1 or CTT2 for therapy might offer new strategies to combat cancer together with conventional surgery and radiation therapies.

6. Conclusions

1. Bisphosphonates are broad-spectrum MMP inhibitors and this inhibition involves cation chelation. Bisphosphonates further exert anti-metastatic, anti-invasive and cell adhesion-promoting properties, which may prevent metastasis formation not only in hard tissues but in soft tissue as well in a dose-dependent manner.

2. Clodronate, at therapeutically attainable concentrations, dose-dependently directly inhibited the activity of the catalytic domain of human recombinant MT1-MMP, reduced the activation of proMMP-2 and down-regulated the expression of MT1-MMP mRNA and protein production by cultured MG-63 human osteosarcoma cells, indicating that clodronate can reduce MT1-MMP RNA expression and activity.

3. In this study, we have discovered and initially characterized two novel peptides, CTT1 and CTT2, which target selectively and specifically gelatinases (MMP-2 and MMP-9), inhibiting the gelatinase activities in many human carcinoma cell lines and endothelial cells in vitro and in vivo. These novel MMPIs may lead to a new treatment options of cancer in the future. The role and involvement of MMPs in pathological conditions demonstrates that the MMPIs are still attractive for drug development.

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