Frauenklinik und Poliklinik der Technischen Universität München,

Klinikum rechts der Isar

(Direktorin: Univ.-Prof. Dr. M. B. Kiechle)

Novel Bi- and Trifunctional Inhibitors of Tumor-associated Proteolytic Systems

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Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Medizin

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. D. Neumeier

Prüfer der Dissertation:

1. Priv.-Doz. Dr. V. Magdolen
2. Univ.-Prof. Dr. Chr. Peschel
3. Univ.-Prof. Dr. H. Graeff, em.

Die Dissertation wurde am 24.09.2003 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 05.05.2004 angenommen.

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Abbreviations

aa	amino acid
APMA	*p*-aminophenylmercuric acetate
APS	ammoniumpersulfate
ATF	amino terminal fragment of uPA
BSA	bovine serum albumin
chCysWT	chicken cystatin wild type
DMEM	Dulbecco's modified Eagle's medium
DNA	desoxyribonucleic acid
DTT	dithiothreitol
E. coli	Escherichia coli
ECM	extracellular matrix
EDC	N-ethyl-N'-(3-diethylaminopropyl)-carbodiimidhydrochloride
EDTA	ethylendiaminotetra acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GuHCl	guanidine hydrochloride
G 418	geneticin 418
h	hour
HEPES	2-[4-(2-hydroxyethyl)-1-piperazin]ethansulfonic acid
HMW	high molecular weight
HRP	horseradish peroxidase
IPTG	isopropyl-ß-D-thiogalactopyranoside
LB	Luria Broth
min	minute
MMP	matrix metalloproteinase(s)
M_r	molecular weight
NHS	N-hydroxy-succinimide
Ni-NTA	Ni^{2+}-nitrilotriacetic acid
OD	optical density
PBS	phosphate-buffered saline

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PCR	polymerase chain reaction
PMA	phorbol-12-myristate-13-acetate
RNA	ribonucleic acid
RT	room temperature
RU	reference unit
SDS	sodium dodecyl sulfate
SPR	surface plasmon resonance
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	tissue factor
TIMP	tissue inhibitor of metalloproteases
TMB	perborate/3,3',5,5'-tetramethylbenzidine
Tris	Tris-(hydroxymethyl)aminomethan
Triton	octylphenolpolyethylenglycol
Tween	polyoxyethylenesorbitan monolaurate
Z-Phe-Arg-NHMec	Z-Phe-Arg-7(4-methyl)coumarylamide

1. Summary

Metastatic disease is the principal cause of death in cancer patients. A positive correlation has been shown to exist between the invasive behavior of tumors and the levels of several proteases including matrix metalloproteinases (MMPs), serine proteases (SP, e.g. the uPA-plasmin system), and cysteine proteases (CPs). Tumor-associated proteases are involved in anti-adhesive, migratory, and proteolytic processes, supporting tumor progression and metastasis. Numerous therapeutic approaches have been used to target various components of protease systems in cancer cells and tissue. Previously, a bifunctional inhibitor was generated by substitution of a loop within the cysteine protease inhibitor chicken cystatin with the uPA receptor (uPAR)-binding site of uPA (chCys-uPA$_{19-31}$; Muehlenweg *et al.,* 2000). This recombinant fusion protein inhibits both, the enzymatic activity of cysteine proteases and binding of uPA to its cell surface receptor uPAR (CD87), the latter representing a crucial step in activating of the uPA/plasmin system. In the present study, we extended this concept and designed bi- and trifunctional inhibitors that are also directed against matrix metalloproteinases (MMPs). For this, the cDNA sequence encoding the N-terminal domain of human tissue inhibitor of matrix metalloproteinase type 1 (TIMP-1) or type 3 (TIMP-3), which harbors the MMP inhibitory activity, was recombinantly fused to the DNA sequence encoding chCys-uPA$_{19-31}$ or wild-type chCys (chCysWT). Recombinant proteins were produced in *E. coli*. As demonstrated by various techniques, these novel fusion proteins effectively interact with and inhibit cysteine and matrix metalloproteinases. Furthermore, trifunctional inhibitors carrying the uPAR-binding site of uPA interfere with binding of uPA to native, cell-associated uPAR. It was demonstrated previously that the bifunctional inhibitor chCys-uPA$_{19-31}$ inhibits cysteine proteases while at the same time interfering with uPA/uPAR-interaction (Muehlenweg *et al.,* 2000). The same is true for the novel bi- and trifunctional inhibitors with respect to their interaction with cysteine proteases and MMPs as shown by surface plasmon resonance technology. Human ovarian cancer cells (OV-MZ-6#8) were stably transfected with expression plasmids encoding the bi- and trifunctional inhibitors. Synthesis and secretion of the inhibitors was proven by a newly developed ELISA, which selectively detects the recombinant proteins. *In vitro* secretion of these inhibitors by stably transfected OV-MZ-6#8 cells did not change the proliferative and adhesive behavior of the transfected cells, with the exception of ovarian cancer cell line OV-N-hTIMP-1-chCys-uPA$_{19-31}$, which showed slightly elevated adhesion to collagen type IV. *In vitro* Matrigel invasion

assays demonstrated that the invasive capacity of cells, endogenously producing the inhibitors, was significantly reduced compared to the vector-transfected control cells. Addition of recombinant bi- and trifunctional inhibitors also reduced invasion of ovarian cancer cells (OV-MZ-6#8) into this extracellular matrix. To test the impact of multifunctional inhibitors on tumor growth *in vivo*, cell lines expressing the various inhibitors were inoculated into the peritoneum of CD1 nude mice and tested for tumor growth and spread compared to vector-transfected control cells. Out of the various combinations, expression of three of these multifunctional inhibitors, namely N-hTIMP-1-chCysWT, N-hTIMP-1-chCys-uPA$_{19\text{-}31}$, and N-hTIMP-3-chCysWT, effected a significant reduction of tumor burden and spread compared to a vector-transfected control cell line. Thus, these novel, compact, and small-size inhibitors directed against two or three different tumor-associated proteolytic systems eventually may represent promising novel cancer therapeutic agents in humans and the respective cDNAs may also be suitable for gene therapy of solid malignant tumors.

2. Introduction

2.1 The Role of Proteases in Tumor Invasion and Metastasis.

To produce metastases, cancer cells have to detach from the primary tumor, invade the extracellular matrix (ECM) and enter into the blood circulation, eventually arrest in the capillary bed, and adhere to subendothelial basement membrane, again to entrance into the organ parenchyma, where they proliferate and induce angiogenesis (Fig. 1). Basement membranes and connective tissue extracellular matrix consist of several major groups of molecules: collagens, fibrin, elastin, glycoproteins and proteoglycans. These ECM constituents are organized and stabilized by a variety of protein-protein and polysaccharide-protein interactions. The ECM is essential for maintaining the structure and organization of tissues, but also restricts cell movement. For cellular invasion to occur, proteolytic degradation of the extracellular matrix components must take a place (Fidler, 1997). The major proteases involved in this degradation process are matrix metalloproteases (MMPs), cysteine proteases (*e.g.* cathepsin B and L), and serine proteases (*e.g.* that of the uPA/plasmin system).

Primary tumor

Blood vessel

ECM

Adhesion of tumor cells to the basement membrane followed by extravasation

Invasion of tumor cells and degradation of the ECM

Formation of metastases
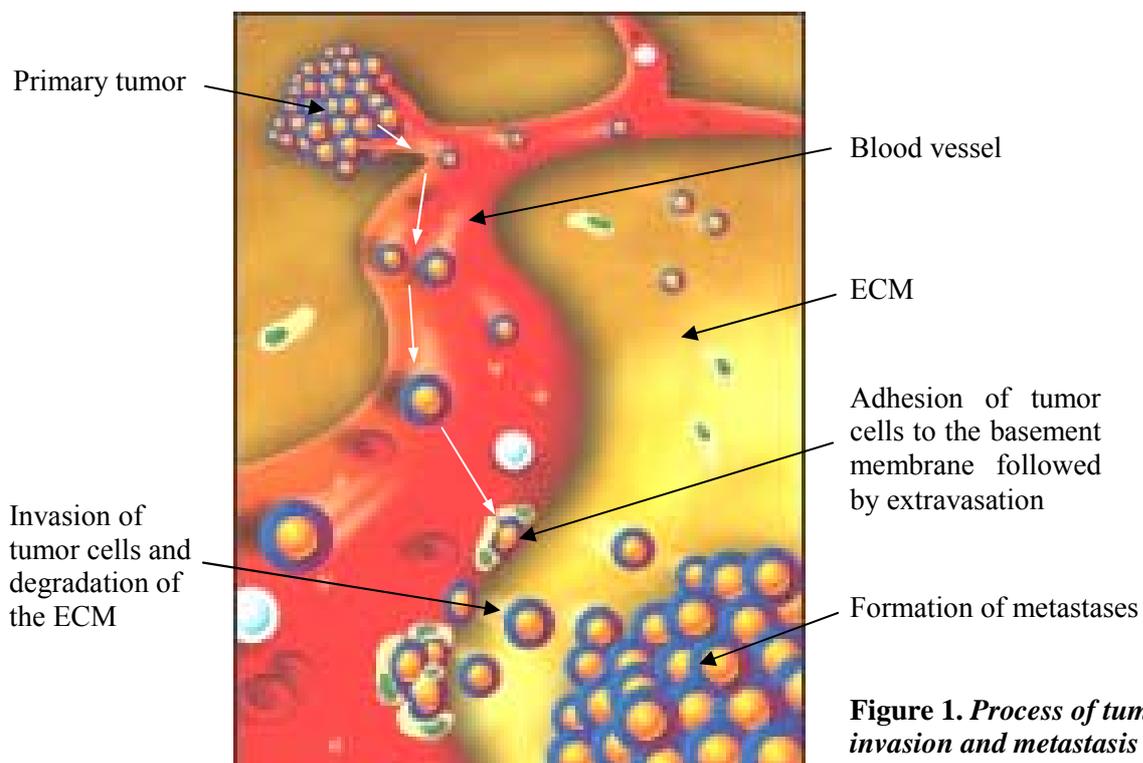
Figure 1. *Process of tumor invasion and metastasis*

2.2 The Plasminogen Activator System

2.2.1 Urokinase-Type Plasminogen Activator (uPA)

uPA is expressed and secreted by a variety of cells (vascular endothelial and smooth muscle cells, epithelial cells, fibroblasts, monocytes/macrophages, and also malignant epithelial cells) as a single-chain polypeptide (scuPA or pro-uPA), consisting of 411 amino acid (aa) residues (M_r: ~ 53,000). pro-uPA is converted into the enzymatically active two-chain form of uPA (HMW-uPA) by cleavage of its peptide bond at K158-I159 by certain proteases such as plasmin, trypsin, plasma kallikrein, and cathepsins B and L (Kobayashi *et al*., 1991; Goretzki *et al*., 1992; Conese and Blasi, 1995, Andreasen *et al*., 1997, Irigoyen *et al*., 1999). HMW-uPA encompasses the C-terminal B chain (aa 159-411), and the N-terminal A-chain (aa 1-158), which are linked by a single disulfide bond at Cys148-Cys279. The B chain harbors the serine protease domain with the amino acid triad His204, Asp255 and Ser356. The A chain consists of the growth factor-like domain (aa 1-49), the kringle domain (aa 50-131) and the interdomain linker region (aa 132-158) (Fig. 2).

A   **B**
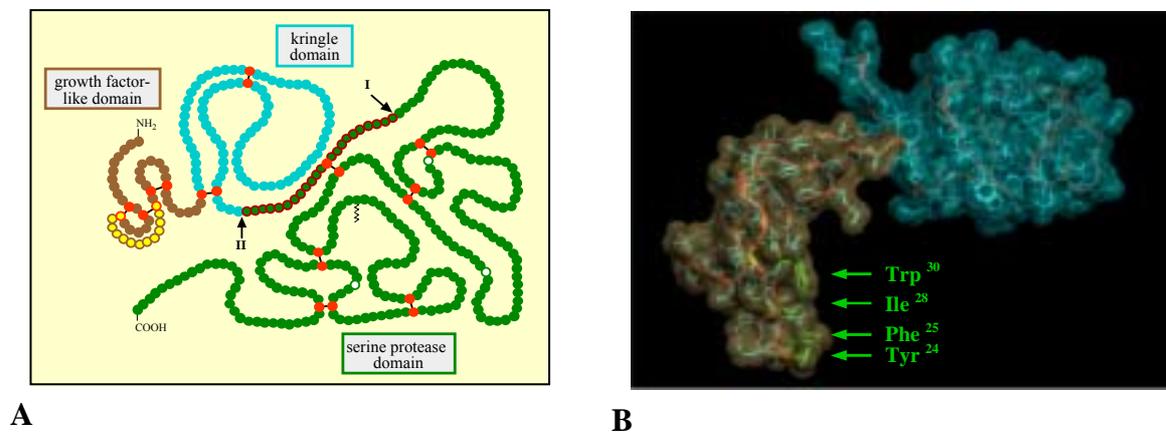
Figure 2. *Structure of uPA*. (A) Schematic representation of uPA. pro-uPA is converted to the enzymatically active two-chain form uPA (HMW-uPA) by cleavage of the peptide bond K158-I159 (arrow I). HMW-uPA consists of a uPAR-binding growth factor-like domain (GFD; brown), a kringle domain (blue), an interdomain linker region and a serine protease domain (green) The uPAR-binding region of uPA within the GFD is depicted in yellow. Proteolytic cleavage between Lys[135]/Lys[136] (arrow II) gives rise to the amino-terminal fragment (ATF) and the low molecular-weight form of uPA (LMW-uPA) **(B)** 3D-structure of the amino-terminal fragment (ATF) of uPA. Semitransparent surface representation of the amino-terminal fragment of uPA (drawn with INSIGHT 2000 using the coordinates from the Brookhaven Protein Database 1URK.pdb). The kringle domain (aa 50-135) is colored in blue, the growth factor-like domain (aa 6-49) is in brown. The uPAR-binding site was previously mapped to aa 19-31 (Appella *et al*., 1987; Magdolen *et al*., 1996; Bürgle *et al*., 1997). The hydrophobic residues important for binding to uPAR are depicted in green, Cys[19] and Cys[31] are marked yellow in this ribbon representation.

The growth factor-like domain is responsible for the interaction of uPA with its receptor uPAR/CD87, whereas the kringle domain mediates interaction of uPA with the extracellular matrix and is involved in the interaction of uPA with its specific inhibitor PAI-1 (Mimuro *et al.*, 1992; Andreasen *et al.*, 2000).

The one-chain zymogen form of uPA (pro-uPA) displays an activity about 250-fold less than that of two-chain uPA. Proteolytic cleavage of uPA in the linker region (Lys135-Lys136) gives rise to the amino terminal fragment (ATF), encompassing the growth factor-like domain (GFD) the kringle domain (Fig. 2), releasing the low molecular weight form of uPA (LMW-uPA) mainly consisting of the serine protease domain of uPA. LMW-uPA does not bind to uPAR, but its proteolytic activity is comparable to the activity of the full-length two-chain urokinase (Andreasen *et al.*, 2000; Stepanova and Tkachuk 2002).

2.2.2 The uPA Receptor (uPAR)

uPAR (CD87) is a single-chain 55-60 kDa glycoprotein and translated into a 313 amino acid polypeptide with a 21 amino acid signal peptide. Due to posttranslational COOH-terminal processing the mature protein comprises about 284 residues. The urokinase receptor lacks a transmembrane sequence and is anchored to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) moiety (Ploug *et al.*, 1991). The GPI anchor is attached to a newly formed carboxy-terminus after removal of a carboxy-terminal sequence from the nascent protein chain. Residue Gly283 is the preferred site at which the cleavage/attachment occurs, but residues Ser282 and Ala 284 may function as alternative sites for GPI attachment (Moller *et al.*, 1992).Mature uPAR protein is highly glycosylated and decreases to a apparent molecular weight of 35 kDa upon deglycosylation (Behrend *et al.*, 1991, 1995; Wang, 2001). Glycosylation is necessary for intracellular transport and maturation of the receptor but can also affect the affinity of the receptor for uPA (Picone *et al.*, 1989; Moller *et al.*, 1992). uPAR consists of three homologous cysteine-rich repeats/domains (D1, D2 and D3) of about 90 amino acids each (Fig. 3). The first domain (aa. 1-87) contains the ligand-binding region, which is involved in high-affinity binding to the growth factor-like module of uPA. The 2nd and 3rd domains, however, are still important for high-affinity uPA-binding as the binding affinity of the isolated first domain is 1,500-fold lower than that of the intact receptor (Ploug and Ellis, 1994; Mazar, 2001).

uPAR has been identified on many cell types, including fibroblasts, smooth muscle cells, endothelial cells, monocytes/macrophages and tumor cells (Nilsen *et al.*, 1988; Blasi *et al.*,

1998, Haddock *et al*., 1991; Tkachuk *et al*., 1996). uPAR displays important functions in relation to directing and localizing the proteolytic activity of uPA. In fact, in the resting cell uPAR is uniformly distributed on the surface, whereas the migrating cell forms clusters of uPAR on the leading edge (Stepanova and Tkatchuk 2002).

Pro-uPA and active uPA bind to uPAR with the same affinity, with a K_d in the subnanomolar range (Andreasen *et al*., 2000). The binding of pro-uPA to uPAR facilitates its activation by proteases including plasmin and at the same time protects bound urokinase from further degradation by plasmin. Thus, uPAR increases the half-life of the functionally active urokinase on the cell surface (Behrend *et al*., 1995).
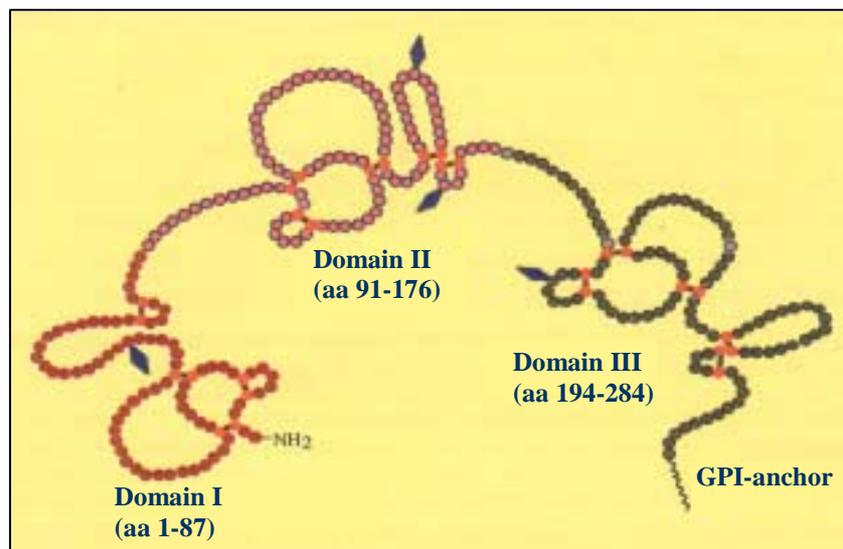
Figure 3. *Schematic representation of uPAR.* uPAR has three homologous domains and is anchored to the membrane by a glycosyl phosphatidylinositol (GPI)-anchor. Disulfide-bridged cysteines are marked in orange. The rhombs (blue) symbolize the identified N-glycosylation sites.

2.2.3 Pericellular Plasminogen Activation

Plasminogen, is converted into the active two-chain serine protease plasmin by proteolytic cleavage of a single peptide bond R560-V561 (Andreasen *et al*., 1997; Irigoyen *et al*., 1999). This cleavage is catalyzed by uPA, t-PA and certain bacterial proteins. Active uPA bound to uPAR catalyzes plasminogen activation much more efficiently than fluid-phase uPA (Andreasen *et al*., 2000). Plasminogen and plasmin can also bind to the cell surfaces of

numerous cell types including tumor cells. Co-expression of plasmin/plasminogen receptor and uPAR would provide a mechanism to generate and localize protease activity at the cell surface (Testa and Quigley, 1990; Felez, 1998).

Cell surface-associated plasmin catalyzes the breakdown of many of the known ECM and basement membrane components, such as fibronectin, laminin, vitronectin, proteoglycans, fibrin, and collagen (Irigoyen *et al*., 1999). These events occur directly by the action of plasmin or indirectly *via* plasmin-dependent activation of other matrix degrading proteases like pro-gelatinase (pro-MMP-2) (Corcoran *et al*., 1996) and pro-stromelysin (pro-MMP-3) (Keski-Oja *et al*., 1992). Plasmin can also activate angiogenic growth factors such as vascular endothelial growth factor (VEGF) or release latent forms of other growth factors, such as hepatocyte growth factor (HGF/SF), fibroblast growth factor 2 (FGF-2) and transforming growth factor β (TGF- β) from ECM (Andreasen *et al*., 2000; Rabbani and Mazar 2001).

2.2.4 Inhibition of the uPA System

Receptor-bound uPA as well as fluid phase uPA rapidly interact with and are inactivated by the inhibitors PAI-1, PAI-2, protease nexin-1, and protein C inhibitor. In contrast, plasmin, which is inactivated in solution by certain inhibitors, is not accessible to serum inhibitors when attached to the cell surfaces (Testa and Quigley, 1990; Andreasen *et al*., 1997). Natural uPA inhibitors are members of the <u>ser</u>ine protease <u>in</u>hibitor superfamily (SERPIN) and belong to the SERPIN-subgroup having an arginine in their reactive inhibitory center. They function by acting as pseudosubstrates and form an irreversible complex with their target protease. PAI-1 is one of the main inhibitors of uPA and is a single-chain glycoprotein of $M_r \sim 50,000$. It is secreted as an unstable active inhibitor, rapidly being converted into its latent form unless it is stabilized by binding to vitronectin. Bound to vitronectin, PAI-1 stays active towards serine protease and can inhibit plasminogen activation by uPA at the cell surface. Upon binding of PAI-1 to uPAR-bound uPA, the ternary complex is rapidly internalized by the cell and degraded. The endocytosis occurs through the interaction with clathrin-coated pit-localized receptors of the LDLR family, either α_2-macroglobulin receptor (α_2MR/LRP), gp330/megalin, or VLDLR (Conese and Blasi, 1995; Stepanova and Tkachuk, 2002). In the endosomes, the uPA-inhibitor complex dissociates from uPAR and is degraded in lysosomes, whereas uPAR is recycled back to the cell surface (Nykjaer *et al*., 1997). Single- and two-chain uPA bound to the α_2MR/LRP can be internalized even in the absence of PAIs, still the presence of inhibitor significantly facilitates endocytosis (Cubellis *et al*., 1990; Nykjaer *et al*.,

1992). Fluid-phase uPA/inhibitor complexes can be internalized also well, but much slower than uPAR-bound uPA/inhibitor complexes (Olson, 1992; Conese, 1995). A surface protein with 200 kDa (p200) together with the α_2MR/LRP was found to be involved in binding and endocytosis of intact uPA and a uPA form lacking the growth factor domain regardless of uPAR (Stepanova and Tkachuk, 2002).

2.2.5 Non-Proteolytic Functions of the uPA System

In recent years, evidence has accumulated, showing that uPA-mediated biological effects relate not only to its fibrinolytic function. Experimental data suggests that uPA binding to uPAR on the plasma membrane activates intracellular signaling systems which regulates cell migration, adhesion, and proliferation.

Cell migration is the locomotion of a cell over an ECM substratum, based on a balance between cell adhesion to the underlying substrate and subsequent loss of cell-matrix contacts. The cell-matrix adhesion at the leading edge is thought to provide guidance and traction for pulling the cell body forward. The necessary mechanical force for this process is generated mainly by contraction of actin filaments. Detachment from the substratum allows retraction at the trailing edge of the cell.

uPA activates a number of signaling pathways that regulate cytoskeleton rearrangement. Varying between cell lines, binding of uPA to uPAR results in activation of Hck kinase, p38, extracellular-signal-regulated kinase (Erk)-2 (Konakova *et al*., 1998), mitogen-activated protein kinase (MAP-kinase), stimulation of phosphorylation of focal adhesion kinase (FAK) (Tang *et al*., 1998), paxillin, p130^{CAS}, and of the DNA-binding activators of transcription STAT-1 and STAT-2 (Koshelnick *et al*., 1997; Dumler *et al*., 1998). Furthermore, occupancy of uPAR by uPA leads to activation of protein kinase Cε (PKCε) and serine phosphorylation of cytokeratins 8 and 18 (Busso *et al*. 1994).

Although there are a number of reports describing signaling effects of uPA, uPAR is a GPI-anchored molecule that does not possess a transmembrane domain. Therefore, uPAR must associate with transmembrane adaptors that are capable to interact with uPAR on one hand and signal to the cytoplasm on the other hand. Possible candidates are integrins, which represent a superfamily of cellular adhesion receptors involved in attachment of cells to extracellular matrix components. Various types of integrins have been reported to interact with uPAR and modulate uPAR-mediated cell interaction with the ECM (Reuning *et al*., 2003). The mode of interaction varies with the integrin type. For example, expression of

uPAR in 293 cells, markedly enhanced β_1 integrin-dependent cell adhesion to vitronectin and significantly blocked that to fibronectin (Wei *et al.*, 1996). In HT1080 fibrosarcoma cells, association of uPAR with β_3 integrins was observed when cells were attached to fibronectin, laminin, or vitronectin. In contrast, association of uPAR with β_1 integrins was observed only when cells were attached to vitronectin (Xue *et al.*, 1997). In pancreatic carcinoma FG cells, uPA induces the interaction of uPAR with $\alpha_v\beta_5$ integrin, leading to cell migration on vitronectin (Yebra *et al.*, 1996).

uPAR is also a high-affinity receptor for the extracellular matrix protein vitronectin. In addition vitronectin binds to urokinase inhibitor PAI-1(Deng *et al.*, 1996). It was shown, that PAI-1 and uPAR display overlapping binding regions on the vitronectin molecule and thus bind competitively. Whereas interaction of the pro-uPA, uPA, ATF, and the uPA-PAI-1 complex with uPAR stimulate its binding to vitronectin (Wei *et al.*, 1994; Irigoyen, 1999), interaction of PAI-1 with uPA markedly diminishes its affinity for vitronectin (Deng *et al.*, 1996). Vitronectin binds also to several integrins expressed on the cell membrane, the binding site is however located at a region within the molecule distinct from the uPAR or PAI-1 binding sites. Importantly, PAI-1 competes for binding of integrins to vitronectin, however uPA binding to PAI-1 suppresses this effect by masking the binding site for vitronectin (Stefansson, 1996; Kjoller, 1997). Thus, by regulating the expression of different components of urokinase-system the migrating cell can switch between cell adhesion and loss of cell-matrix contacts.

Another function of the uPA/uPAR system relates to its chemotactic properties. Upon ligand binding (such as pro-uPA, uPA or ATF), uPAR undergoes either a conformational change or a proteolytic cleavage, exposing a chemotactic epitope located in the linker region connecting domains D1 and D2. It was shown that the uPAR-induced chemotactic effect requires the transient activation of Src and Janus-kinases (Resnati *et al.*, 1996, Fazioli *et al.*, 1997, Blasi and Carmeliet, 2002).

The uPA system is also involved in cell proliferation. Different structural requirements within the uPA molecule for different cells have been reported for its growth factor-like activity. Nevertheless, in many cases, binding of uPA to its receptor was a critical point for induction of cell proliferation (Reuning *et al.*, 1998).

The evidences for the involvement of uPA system in angiogenesis is provided in part by localization studies. It was found that uPA, uPAR and PAI-1 are expressed during angiogenesis *in vivo* in a variety of settings, whereas no expression was detected in quiescent endothelium (Pepper *et al.*, 1996). Both uPAR and the catalytic activity of uPA have been

demonstrated to play a role in endothelial cell migration and differentiation. uPA itself had a proangiogenic effect, when assayed in the rabbit cornea or on chicken chorioallantoic membrane (Berman *et al*., 1982; Goldfarb *et al*., 1986; Rabatti *et al*., 1999). Studies in PAI-1 null mice have revealed an absolute requirement for PAI-1 in tumor-induced angiogenesis. In several murine models blocking of the uPA/uPAR interaction (e.g. by ATF) impaired neovascularisation (Tkachuk *et al*., 1996; Pepper, 2001; Rabbani *et al*., 2001).

2.3 Matrix Metalloproteinases

The matrix metalloproteinases (MMPs), also known as the matrixins, represent one group of proteases that are involved in ECM degradation. They are zinc (Zn)-containing endopeptidases that have optimal activity at neutral pH. Most of these enzymes are secreted by a variety of connective tissue cells, including fibroblasts, osteoblasts, chondrocytes and endothelial cells, as well as inflammatory cells such as macrophages, neutrophils and lymphocytes. To date, 24 mammalian MMPs have been identified and, according to substrate specificities and structural homologies, can be subdivided into 5 groups: collagenases, stromelysins, gelatinases, membrane-type MMPs (MT-MMPs) and other MMPs (Johansson *et al*., 2000; Stetler-Stevenson and Yu, 2001). MMPs have a characteristic multidomain structure consisting of (i) a *signal peptide*, (ii) a *pro-peptide*, which is essential for maintaining the pro-MMP in a latent form, (iii) a *catalytic domain* containing the highly conserved Zn^{2+} -binding site (HExGHxxGxxHS/T), (iv) a prolinerich *hinge region* that links the catalytic domain to (v) the *hemopexin-like* domain, which determines the substrate specificity of the MMPs. In addition, the catalytic domain of gelatinases contains three repeats of the *fibronectin-type II domain*, involved in binding of these enzymes to gelatine. MT-MMPs contain a short *transmembrane* domain of ~20 hydrophobic amino acids in the C-terminal end of the hemopexin domain followed by a 24 amino-acid intracellular domain (Nagase and Woessner, 1999; Overall and Lopez-Otin, 2002).

The importance of these proteases is based on their ability to collectively degrade all structural components of the extracellular matrix and basement membrane comprising collagens, laminins, fibronectin, vitronectin, and heparansulfate proteoglycans (Yu *et al*., 1997; Johansson *et al*., 2000; Pupa *et al*., 2002). Degradation of ECM by MMPs leads to release of ECM-sequestered growth factors, that play an important role in tumor cell survival and proliferation as well as angiogenesis. Some of these growth factors, including transforming growth factor β (TGF-β), insulin-like growth factors (IGF-I and II) and basic

fibroblast growth factor (bFGF) are maintained in latent form in complex with various binding proteins, others, including heparin binding epidermal growth factor (HB-EGF), TGF-α and TNF-α are expressed in a pro-form on the cell surface. There is increasing evidence that MMPs not only help release ECM sequestered growth factors but proteolytically activate a variety of latent ECM- and cell membrane-bound growth factors and cytokines (Stamenkovic, 2000; Chang and Werb, 2001; Hornebeck *et al*., 2002; Pupa *et al*., 2002). MMPs were also shown to cleave some adhesion receptors (CD44, E-cadherin) and release them from the cell-surface, thus promoting cell invasion (Kajita *et al*., 2001; Davies *et al*., 2001; Noe *et al*., 2001).

MMPs are also implicated in the promotion of angiogenesis. A number of studies showed that MMP inhibitors reduces the angiogenic response *in vitro* and *in vivo*. Possible mechanisms of MMPs action include (i) degradation and rebuilding of the ECM in the area surrounding the proliferating endothelial cells and (ii) release and activation of pro-angiogenic factors (Kurschat and Mauch, 2000; Pepper, 2001; John and Tuszynski, 2001; Yana and Seiki, 2002). Interestingly, MMP-3, MMP-7, MMP-9 and MMP-12 have recently been shown to generate angiostatin from plasminogen, indicating that they may also serve to limit tumor-induced angiogenesis (Dong *et al*., 1997; Patterson *et al*., 1997; Lijnen *et al*., 1998).

Most MMPs are secreted as latent precursors (zymogens), which are proteolytically activated in the extracellular space by a number of proteases, including plasmin and other MMPs.

The activity of MMPs is controlled both by proenzyme activation and inhibition by nonspecific inhibitors, e.g. α1-protease inhibitor and α 2-macroglobulin and by specific tissue metalloproteases inhibitors, the TIMPs (Yu *et al*., 1997; Stamenkovic, 2000; Johansson, 2000).

2.4 Tissue Inhibitors of Matrix Metalloproteinases (TIMPs)

Currently four members of the TIMP-family (TIMP-1, -2, -3 and -4) are known and characterized (Brew *et al*. 2000). They are small proteins with a molecular weight between 21 and 28 kDa and are secreted by many different cell types. Each TIMP consists of two distinct structural and functional domains: an N-terminal and a C-terminal domain, each stabilized by 3 disulfide bonds. TIMPs have 12 conserved cysteine residues, contributing to the secondary structure and their ability to inhibit MMPs (Birkedal-Hansen *et al*., 1993; Bodden *et al*., 1994). TIMP-1 is glycosylated (8-9 kDa), also TIMP-3 can contain sugar components up to 7 kDa (Apte *et al*., 1995); TIMP-2 and TIMP-4 are non-glycosylated. TIMP-1, TIMP-2 and

TIMP-4 are present in soluble form, whereas TIMP-3 is not soluble, but tightly bound to the extracellular matrix. This interaction is mediated by the C-terminal domain of the inhibitor (Langton *et al.* 1998).

The N-terminal domain of the TIMPs harbors the inhibitory activity by forming a tight 1:1 non-covalent complex with the catalytic center of active MMPs (Fig.4). Recombinant truncated TIMPs containing only the N-terminal domain retain most of their inhibitory activity towards MMPs (Huang *et al.* 1997).

Figure 4. *Ribbon representation of the TIMP-1/MMP-3 complex structure (pdb-code: 1UEA)*. The N-terminal domain of TIMP-1 (residues 2-126) is colored in yellow, the C-terminal domain in magenta; MMP-3 is colored in green, catalytic zinc in red, calcium in blue and selenium in orange. Only the N-terminal domain of the TIMP-1 molecule performs interactions to the MMP-3 protein in the complex. The figures were drawn based on the coordinates from the Brookhaven Protein Database using the computer program INSIGHT II (Molecular Simulations Inc.).

Although different TIMPs bind tightly to most MMPs, some differences in their inhibitory properties have been reported. Thus, TIMP-2 and TIMP-3, unlike TIMP-1, are effective inhibitors of the membrane-type MMPs (MT-MMPs), while TIMP-3, but not TIMP-1, -2 or -4, is a good inhibitor of tumor necrosis factor-α converting enzyme (TACE), a metalloproteinase that is not a member of the matrixin family, but belongs to the ADAM family (Murphy *et al.*, 1995, Amour *et al.*, 1998).

Besides their inhibitory role, TIMPs seem to have other functions. TIMPs differ in the types of non-inhibitory complexes that they form, mediated by their C-terminal domains. Thus, TIMP-2 binds tightly to pro-MMP-2 forming a complex that is important in the cell-surface activation of pro-MMP-2, while TIMP-1 forms a specific complex with pro-MMP-9 (Goldberg *et al.*, 1989; Goldberg *et al.*, 1992).

Several studies have demonstrated a role for TIMPs in the regulation of apoptosis. TIMP-1 and TIMP-2 have been found to have anti-apoptotic properties and, even more, growth promoting activities for many cell lines (Bertaux *et al.*, 1991; Hayakawa *et al.*, 1992 and

1994; Valente *et al.*, 1998; Mannello and Gazzanelli, 2001). On the contrary, TIMP-3 overexpression was associated with induction of apoptosis. This apoptotic effect was associated with a reduced capacity of TIMP-3-transduced cells to bind to ECM components suggesting disruption of cell-matrix survival pathway (Ahonen *et al.*, 1998; Baker *et al.*,1999; Fassina *et al.*, 2000). The role of TIMPs in angiogenesis is supported by the observation that they are able to inhibit new vessel formation in bioassays. TIMPs may affect angiogenic processes in several ways: (i) preventing MMP-mediated endothelial cell detachment and migration, (ii) blocking the release of matrix-bound angiogenic factors and (iii) preventing degradation of the ECM. Several studies have noted a role for TIMPs in the regulation of apoptosis. TIMP-1 and TIMP-2 have been found to have anti-apoptotic effects on many cell lines. On the contrary, TIMP-3 have been shown to induce apoptotic cell death (Valente *et al.*, 1998; Baker *et al.*, 1999; Brew *et al.*, 2000; Fassina *et al.*, 2000). The above observations indicate that TIMPs are multifunctional proteins, but the mechanisms underlying these effects are not yet fully understood.

2.5 Cysteine Proteases

Cysteine proteases, synonymous with thiol proteases, are small proteins with molecular weights varying from 23 to 30 kDa with the exception of cathepsin C, which an oligomeric enzyme with a $M_r \sim 200,000$. Most cysteine proteases are evolutionary related to the structurally well-defined cysteine protease papain and are therefore called papain-like cysteine proteases (Turk *et al.*, 2000; Grzonka *et al.*, 2001). Enzymatic activity of cysteine proteases is related to the presence of a catalytic diad formed by Cys25 and Hisl59, which is involved in the hydrolytic reaction. Cysteine proteases catalyze the hydrolysis of various polypeptide substrates and are most active under reducing and mildly acidic conditions (pH 5 - 6.5). The human cysteine proteases of this family are mainly localized in lysosomes, the most important being the cathepsins B, H, L and S (Barrett and Kirschke, 1981; Barrett *et al.*, 1988). Lysosomal cathepsins are synthesized as inactive precursors and are activated by proteolytic removal of the N-terminal pre-peptide during the passage to the endoplasmic reticulum (McGrath, 1999).

Cathepsins B, H and L have a broad substrate specificity compared to other lysosomal enzymes (such as the aspartic protease cathepsin D) and play key roles in the intracellular degradation of proteins and peptides (Kominami *et al.*, 1991; Mort *et al.*, 1997). Besides their role inside lysosomes, cysteine proteases degrade proteins outside lysosomes, and also

process other proteins outside lysosomes (Jutras *et al.*, 1999; Turk *et al.*, 2000; Turk *et al.*, 2001). Lysosomal enzymes were found to be present in the extracellular/extralysosomal environment in the pro-forms, which are substantially more stable than the mature enzymes (Mason *et al.*, 1987). Although these enzymes show maximal activity at mildly acidic conditions, there are reports of hydrolytic activity at neutral pH (Buttle *et al.*, 1988). Under some pathological conditions lysosomal enzymes can be secreted or released by autolysis that will lead to irreversible damage of the tissue. Recent studies have shown that cathepsins B, H and L are involved in cancer progression either by direct degradation of extracellular matrix components (type I and IV collagen, laminin) or by activation of other proteases, such as uPA (Schwartz, 1995; Mort *et al.*, 1997; Lah and Kos, 1998; Koblinski *et al.*, 2000; Mai *et al.*, 2000; Schmitt *et al.*, 2000). Moreover, cathepsin B was found to be involved in inactivation of TIMPs. Indeed, treatment of TIMP-1 and TIMP-2 with cathepsin B results in their fragmentation and loss of MMP-inhibitory activity (Kostoulas *et al.*, 1999).

There are several ways in which the enzymatic activity of lysosomal cysteine proteases is regulated, two of the most important are regulation of proenzyme activation and inhibition by their endogenous inhibitors, unspecific like α_2-macroglobulin and specific like cystatins.

2.6 Cysteine Protease Inhibitors, Cystatins

Cystatins comprise single-chain inhibitory proteins that reversibly inhibit the activity of the cysteine proteases, which are widely distributed in the human body (Turk and Bode, 1991; Barrett, 1986; Grubb *et al.*, 2000). Three types of cystatins are present in vertebrates: type-1 cystatins are synthesized without a signal peptide and, thus, are normally present in the cell (cystatin A and B, also named stefin A and B). The two other types are secretory type-2 single-domain cystatins (C, D, M/E, F, S, SN, SA) and type-3 multi-domain cystatins (high and low molecular weight kininogens). The type-1 cystatins (approx. 100 aa; $M_r \sim$ 11-12,000), lack both disulfide bridges and carbohydrate groups. Type-2 cystatins (e.g. chicken cystatin and human cystatin C) are molecules of about 120 aa ($M_r \sim$ 13-14,000) and are characterized by two intra-chain disulfide bonds located towards the C-terminus. With the exception of rat cystatin C, type-2 cystatins are non glycosylated (Esnard *et al.*, 1990). Type-3 cystatins encompass three type-2 cystatin-like domains, which very likely resulted from gene duplications (Müller-Esterl *et al.*, 1985). They contain additional disulfide bonds and are glycosylated.

Cystatins form equimolar, tight and reversible complexes with papain-like cysteine proteases (Barrett *et al*., 1986). There are three well-conserved regions in the cystatin superfamily that have been implicated in cysteine protease inhibition (Fig. 5). These regions are (i) a region near the N-terminus, (ii) a first hairpin loop containing the highly conserved sequence Gln-Xaa-Val-Xaa-Gly, and (iii) a second hairpin loop containing a Pro-Trp pair (Abrahamson, 1994; Bode and Huber, 2000). All three regions contain many hydrophobic residues, indicating that hydrophobic interactions play an important role in the interaction of cystatins with target molecules. Based on structural theory, these three regions penetrate the active site of the enzyme in such a way that the papain active site Cys25 residue is blocked (Bode and Huber, 2000). An additional reactive site in the loop between the α-helix and the first strand of the main β-pleated sheet with its Asn39 residue was detected and shown to be responsible for the inhibition of mammalian legumain by some cystatins (Alvarez-Fernandez *et al*., 1999). In mammals, cystatins are found in relatively high concentration in many biological fluids, such as seminal plasma, cerebrospinal fluid, plasma, saliva and urine. Cystatin A, B, and C are present in various cells and tissues (Dickinson *et al*., 1993; Abrahamson *et al*., 1994), whereas cystatin D, S, SN, and SA are almost limited to saliva, tears and seminal plasma (Isemura *et al*., 1984; Grubb *et al*., 2000). Kininogens are major plasma proteins, involved in the tonus regulation of blood vessels and coagulation in addition to its function as cysteine endopeptidase inhibitor (Barrett *et al*., 1986).

A large number of normal and pathological processes are controlled by the balance between cysteine proteases and their inhibitors. Uncontrolled proteolysis of human cysteine proteases can lead to irreversible damage such as inflammatory diseases, neurological disorders, infection and tumor metastases (Henskens *et al*., 1996, Kos *et al*., 1998, Hirai *et al*., 1999, Kos *et al.*, 2000a).
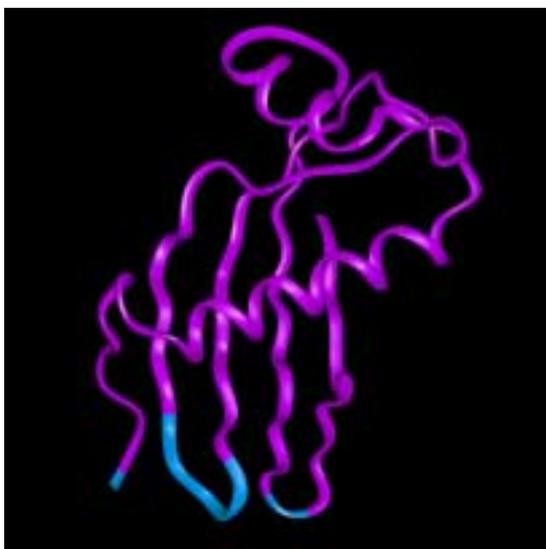
Figure 5. *Ribbon representation of the structure of chicken cystatin (pdb-code: 1CEW).* The residues which are essential for inhibition of cysteine proteases are distributed on three different adjacent loops and are colored in blue. The figures were drawn based on the coordinates from the Brookhaven Protein Data-base using the computer program INSIGHT II (Molecular Simulations Inc.).

2.7 Clinical Relevance of Proteolytic Enzymes and Their Inhibitors

A positive correlation has been shown to exist between the invasive behavior of tumors and the levels of all three here described groups of proteases. Already in the 1970's, E. Reich and collaborators showed, that increased secretion of plasminogen activator (PA) by tumor cells is largely responsible for the enhanced proteolytic activity associated with malignances (Reich, 1978). Following this early observation, a number of subsequent studies reported on the significant correlation between elevated level of uPA in primary tumor and cancer progression. In a variety of malignancies, uPA and PAI-1 antigen emerged as a strong, independent, statistically significant prognostic factor for both disease-free and overall survival. Increased expression of uPA receptor in the primary tumor also correlates with a highly invasive phenotype and poor prognosis in several tumor types (Fig.6). (reviewed by Andreasen *et al*., 1997; Reuning *et al*., 1998; Irigoyen *et al*., 1999; Schmitt *et al*., 2000; Mazar, 2001). In addition, expression of uPAR and uPA in disseminated cancer cells in bone marrow of patients with gastric and breast cancers, respectively, predict an early relapse (Heiss *et al*., 1995; Solomayer *et al*., 1997).
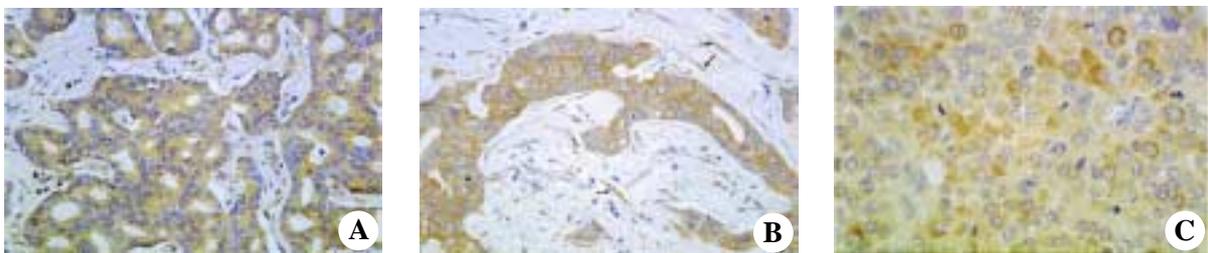
Figure 6. *u-PA, uPAR, PAI-1 expression in breast cancer.*(A) Immunoperoxidase staining with diaminobenzidine as the brown chromogenic substrate showing strong uPA expression in a grade I invasive ductal carcinoma; **(B)** strong uPAR expression in the tumor cells and fibroblasts of a grade III ductal carcinoma; **(C)** strong PAI-1 expression in a grade III ductal carcinoma.(taken from Dublin *et al.* Am. J. Pathol. 2000, 157:1219-1227).

A number of studies have demonstrated a positive correlation between MMP expression and invasive and metastatic potential of malignant tumors, including colon, lung, head and neck, basal cell, breast, thyroid, prostate, ovarian, endometrial and gastric carcinomas (Fig. 7) (Yu *et al*., 1997; Shapiro, 1998; Kähäri and Saarialho-Kere, 1999; Curran and Murray, 1999). In several cancer types high MMP levels can also predict the recurrence of tumors and relate to poor survival (Gohji *et al*., 1998; Kanayama *et al*., 1998; reviewed by Vihinen and Kähäri. 2002).
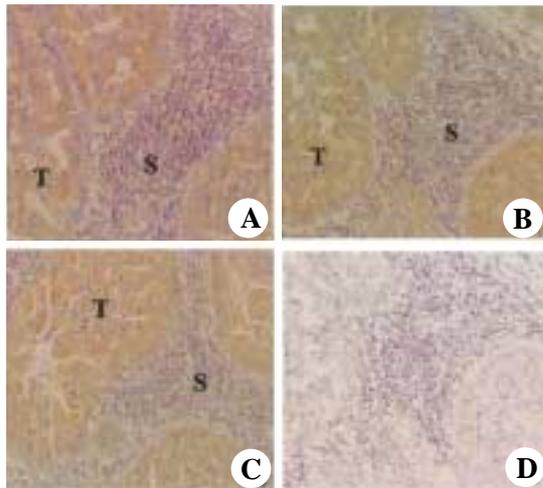
Figure 7. *Immunohistochemical detection of matrix metalloproteinase (MMP)-2, MMP-9 and membrane type 1 (MT1)-MMP in endometrial carcinoma tissue.* **(A)** Matrix metalloproteinase-9 predominantly immunolocalized to tumor tissue (T) in grade 2 carcinomas. Variable stromal (S) staining is evident particularly in areas adjacent to tumor tissue. **(B)** Matrixmetalloproteinase-2 immuno-localized to tumor tissue and to the stromal compartment to varying degrees in grade 2 carcinoma. **(C)** Membrane type 1-MMP immuno-localization predominantly paralleled that of MMP-2. **(D)** Negative control. (taken from Di Nezza *et al*. Cancer. 2002, 94:1466-1475).

In malignant tumors, most MMPs are produced by nonmalignant cells rather than tumor cells. Tumor cells can also secrete factors, such as the extracellular MMP inducer (EMMPRIN), which enhance the expression of MMPs by fibroblasts (Guo *et al*., 1997; Yu *et al*., 1997; John and Tuszynski, 2001). In addition, many growth factors and cytokins secreted by tumor-infiltrating inflammatory cells as well as by tumor cells are capable to influence the stromal expression of MMPs. Although *in vitro* and *in vivo* experiments showed that TIMPs have an anti-tumor and anti-metastatic effect, the majority of the recent clinical studies have stated a positive correlation between TIMP levels and poor outcome in individual types of tumors (reviewed by Curran and Murray, 1999; McCarthy *et al*., 1999; Remacle *et al*., 2000). There are several scenarios to understand why the elevated TIMP expression is associated with malignant cancer cells: (i) the high level of TIMP-1 and TIMP-2 may favor tumor growth in an MMP-independent manner through their growth promoting and anti-apoptotic effects; (ii) TIMP-2 is known to be involved in pro-MMP-2 activation; and (iii) this increased expression of TIMPs may represent one of the subsequent acute host responses to the elevated MMPs activity and try to balance the local tissue degradation. However, the MMP/TIMP ratio was not determined in these studies and evaluation of either TIMP or MMP expression alone is likely not sufficient for prognostication of malignancies. Thus the balance of MMP-9 and MMP-2 to TIMP- I and TIMP-2 expression was shown to be an essential factor in the aggressiveness of cervical cancer (Nuovo *et al*., 1995). In this study, the ratios of MMP-9 and MMP-2 expression to TIMP-1 and TIMP-2 were approximately 1 in those cancers with a good prognostic factors and 3.4 to 5.4 for patients with a poor prognostic factors.

Alteration in the cysteine protease/cysteine protease inhibitors (CPI) ratios have been postulated to contribute to the malignant progression (Sloane 1990; Kane and Gottesman,

1990). This imbalance can be caused by increased activity of the cysteine protease as well as by decrease in the concentration and/or activity of CPIs. Cysteine proteases, in particular cathepsin B, were already shown in the early 1980s to be associated with malignancy (Poole *et al.*, 1980; Sloane *et al.*, 1981). The increased secretion and activity of cathepsin B and L was shown positively correlate with invasive and metastatic capabilities of chondrosarcoma, brain, prostate, breast, and gastrointestinal cancer (Fig. 8) (Berquin and Sloane, 1996; Keppler *et al.*, 1996; Yan *et al.*, 1998; Frosch *et al.*, 1999). Cathepsin B and L were also described as prognostic markers in some malignancies (Schwartz, 1995; Harbeck *et al.*, 2001).
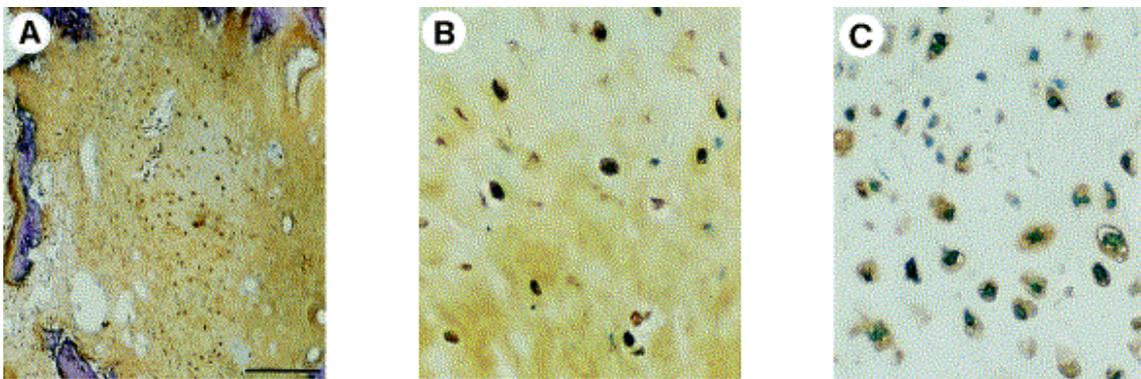
Figure 8. *Expression of cathepsin L in human chondrosarcoma.* Cathepsin L (brown precipitate) was detected by immunohistochemistry using the avidin-biotin complex method. In grade 1 **(A, B)** and grade 2 **(C)** chondrosarcomas most of the chondrocytes are positive for cathepsin L. The sections were counterstained with hematoxylin (taken from M. Soderstrom *et al.* Matrix Biology. 2001, 19:717-725).

Cystatin C is the strongest inhibitor of cysteine proteases, it was therefore most frequently investigated in tumor invasion and metastasis. Tumor-associated expression of cystatin C was at first detected in the ascitic fluid from patients with ovarian cancer (Lah *et al.*, 1990). The increased level of cystatin C in the blood of patients with breast cancer (Lah *et al.*, 1992), fibrosarcoma (Corticchiato *et al.*, 1992), melanoma (Kos *et al.*, 1997), colorectal carcinoma (Kos *et al.*, 2000a), and lung cancer is associated with the progression of the malignant disease (Kos *et al.*, 2000b). At the same time cathepsin B/cystatin C complexes were found to be less abundant in the blood of patients with malignant tumors than in healthy controls indicating an imbalance between cysteine proteases and cystatin C in cancer cells (Zore *et al.*, 2001). An elevated concentration of the latent (inactive) fraction of cystatins was determined in the blood of patients with head and neck cancer and in the urine of patients with colorectal cancer (Siewinski *et al.*, 1992 and 1994). The significant decrease of the inhibitory activity of

cystatins in biological fluids in cancer patients may be taken as a further support to the assumption of an involvement of cysteine proteases in tumor progression and metastasis (Lah and Kos, 1998).

2.8 Interaction of Proteolytic Systems

Serine proteases, matrix metalloproteases and cysteine proteases do not act as isolated systems, but interact and by this efficiently activate each other (Fig. 9). An important step in activation of proteolytic cascade is the binding of pro-uPA to its specific receptor that facilitates the conversion of inactive single chain uPA to its active two-chain form by proteases including plasmin and cathepsin B and L. Activation of uPA leads to generation of plasmin from plasminogen. Plasmin is a broad substrate range serine protease that not only catalyses the breakdown of many of the known extracellular matrix proteins, but is also involved in the proteolytic activation of pro-uPA and pro-MMPs. The latter can in turn activate other MMPs and degrade structural components of the extracellular matrix and basement membrane.
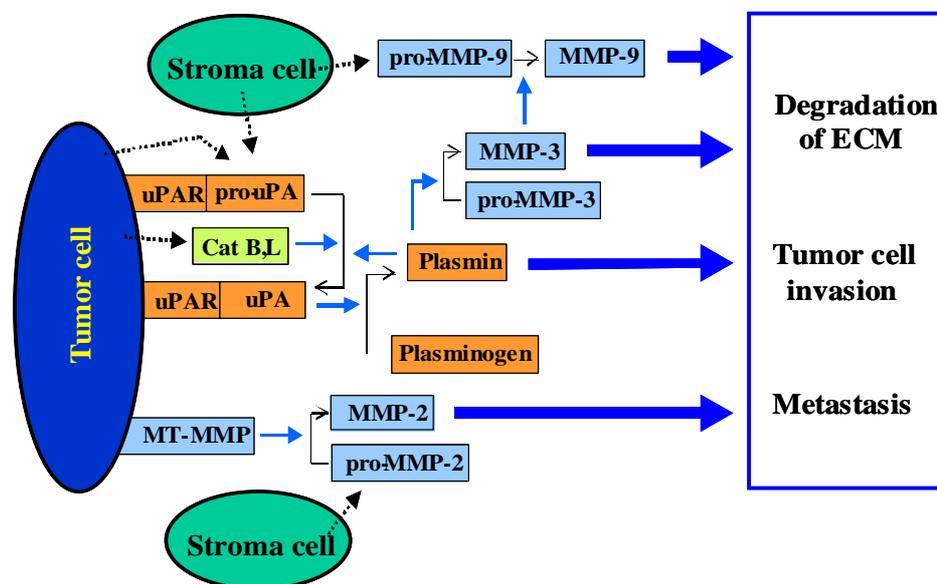
Figure 9. *Interaction of tumor-aassociated proteolytic systems*

2.9 Proteolytic Systems as Therapeutic Target

In view of their important role in tumor invasion and metastasis, MMPs, cysteine proteases and uPAR/ uPA/plasmin system have become promising therapeutic targets for the development of new anti-tumor drugs. Numerous approaches have been used to validate uPAR as a target for cancer therapy. Many of the studies have focused on the development of substances which block uPA-uPAR interaction. Different peptide and small molecule antagonists, derived from uPAR binding region of uPA, were tested. Kobayashi *et al.* (1994) used linear peptides spanning amino acids 17-34 of mouse uPA to inhibit lung metastasis in a Lewis lung model. Ploug *et al.* (2001) developed a 9-mer linear peptide antagonist of the uPA/uPAR interaction that could inhibit cancer cell intravasation in a chicken chorioallantoic membrane assay. Identification of the amino acids critical for binding of uPA to uPAR located the minimal uPAR-binding region of uPA to uPA_{19-31} (Fig. 10A) (Appella *et al.*, 1987; Magdolen *et al.*, 1996, Burgle *et al.*, 1997). The short distance between Cys19 and Cys31 in the native molecule allowed cycling of the linear uPA_{19-31} using disulfide bridge formation giving rise to the cyclic peptides such as $cyclo^{19,31}uPA_{19-31}$ and $cyclo^{21,29}uPA_{21-30}$ (Fig. 10B,C) (Burgle *et al.*, 1997; Magdolen *et al.*, 2001). These cyclic peptides were able to compete with the binding of uPA to uPAR on cells with an IC_{50} ~10-20 nM and are effective in reducing tumor growth *in vivo*. $Cyclo^{19,31}uPA_{19-31}$ reduced the growth of human MDA-MB231 breast cancer cell and the cyclic peptide $cyclo^{21,29}uPA_{21-30}$ effectively reduced tumor growth and spread of human ovarian cancer cells in a mouse tumor model (Sperl *et al.*, 2001; Sato *et al.*, 2002).

A   **B**   **C**
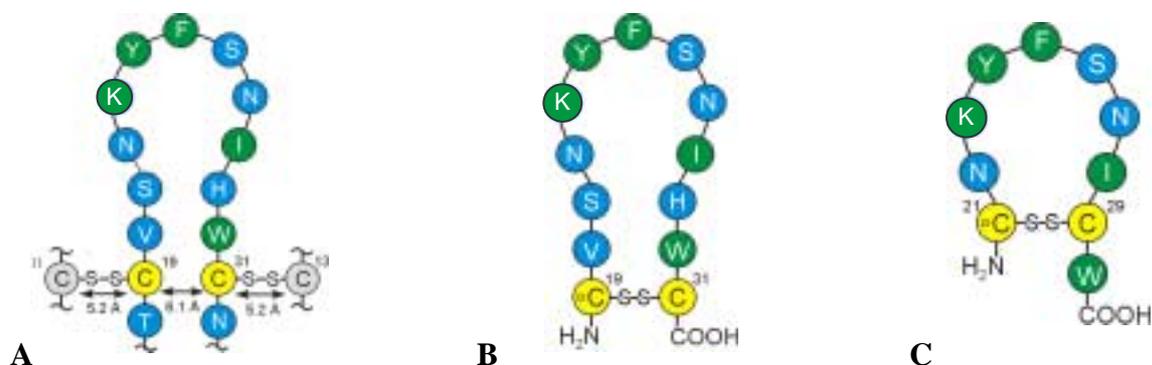
Figure 10. Synthetic peptides derived from the uPAR-binding site of uPA. (A) Structure of uPAR-binding region of uPA (B) Structure of $cyclo^{19,31}uPA_{19-31}$ and (C) $cyclo^{21,29}uPA_{21-30}$. Using alanine mutational analysis, Cys19, Cys31 (colored in yellow) and Lys23, Tyr24, Phe25, Ile28, and Trp30 (colored in green) were identify to be critical for binding to uPAR.

Down regulation of uPAR expression has also been achieved in pre-clinical models using anti-sense and gene therapy approaches leading to increased tumor dormancy, suppression of tumor growth and metastasis development (Kook *et al.*, 1994; Li *et al.*, 1998 and 1999; Aguirre Ghiso *et al.*, 1999, Mohan *et al.*, 1999). Another approach, that has been successfully used, was transfection of cancer cells with expression vectors harboring cDNAs encoding catalytically inactive uPA or soluble uPAR (suPAR). In these models, enzymatically inactive uPA works as an antagonist of uPA and suPAR is functioning as a scavenger for uPA. In both cases, a significant inhibition of uPA/uPAR interaction and an anti-tumor effect could be observed (Crowley *et al.*, 1993; Krüger *et al.*, 2000; Lutz *et al.*, 2001). An effect in diminishing tumor cell adhesion, invasion, and metastasis was also achieved using antibodies to uPA or uPAR (Ossowski and Reich, 1983; Luther *et al.*, 1997; Rabbani and Gladu, 2002).

A number of studies has shown that gene delivery of natural inhibitors of matrix metalloproteinases into malignant cells is a potent way of inhibiting tumor invasion. However the limited clinical utility of this approach and the inability to mass produce natural TIMP led to the development of synthetic MMPIs. Several synthetic MMP inhibitors have been introduced in clinical trials and have been expected to represent a new approach to cancer treatment in addition to traditional cytotoxic drugs. These inhibitors have shown efficacy in preclinical tumor model system. However, some of them failed in phase II or III of clinical trials, because of the absence of marked responses and unpleasant side effects due to their broad substrate specificity. Currently more specific MMP inhibitors have been synthesized and are now in phase I/II clinical trials for various forms of cancer (Wojtowicz-Praga *et al.*, 1997; John and Tuszynski, 2001; Vihinen and Kähäri 2002; Coussens *et al.* 2002).

Cystatin C activity is also correlated to the malignant phenotype as shown by *in vitro* and *in vivo* tumor model systems. Transfection of cystatin C cDNA into B16 melanoma cells led to an inhibition of tumor cell invasion through an artificial matrix barrier *in vitro* and to a significant reduction of the number of lung metastases after the injection into the tail vein of nude mice (Sexton and Cox, 1997, Cox *et al.*, 1999). The inhibitory effect of cystatin C on tumor cell invasion was also demonstrated in *in vitro* Matrigel invasion assays using transfected murine SCC-VII squamous carcinoma cells, ras-transformed breast epithelial cells, human fibrosarcoma, and colon carcinoma cell lines (Corticchiato *et al.*, 1992; Coulibaly *et al.*, 1999; Premzl *et al.*, 2001).

2.10 Objective

Since matrix metalloproteinases, cysteine proteases, and the uPA/plasmin system play an important role in tumor invasion and metastasis and to some extent display overlapping, redundant activities, it seems an interesting strategy to modulate their proteolytic activity simultaneously. Recently, bifunctional inhibitors were designed, which on the one hand are directed against the enzymatic activity of cysteine proteases, on the other inhibit binding of the serine protease uPA to its receptor (uPAR) (Muehlenweg *et al.,* 2000).

In connection with these findings, the aim of the present study was:

1) to extend this concept and generate bi- and trifunctional inhibitors, also directed against MMPs;

2) to develop suitable expression and purification systems;

3) to biochemically analyze these multifunctional inhibitors with respect to their inhibitory activity against MMPs, cysteine proteases, and uPA/uPAR-interaction;

4) to test the biological effect of exogenously added or endogenously produced and secreted multifunctional inhibitors on *in vitro* tumor cell proliferation, adhesion, and invasion of human ovarian cancer cells;

5) to analyze the effects of these multifunctional inhibitors *in vivo* on primary tumor growth and spread of human ovarian cancer cells in a *xenograft* nude mouse model.

3. Materials and Methods

3.1 Materials

3.1.1 Cell Lines

The ovarian cancer cell line OV-MZ-6 was established from ascitic fluid of a patient with a serous-papillary ovarian carcinoma FIGO IV (Möbus *et al*., 1992). This cell line was subcloned in the clinical research group of the "Frauenklinik der TU München", characterized and further cultured as OV-MZ-6#8 (Fischer *et al.,* 1998; Lutz *et al.,* 2001).

The lymphoma cell line U937 was established from a pleural effusion of a patient with diffuse lymphoma (ATCC, Rockland, USA)

3.1.2 *E. coli* Bacterial Strain

E. coli strain M15[pREP4] (Qiagen, Hilden, Germany) derived from the K12 family displays the phenotype Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+. This strain contains the repressor pREP4 plasmid which confers kanamycin resistance and constitutively expresses the *lac* repressor protein. In combination with the pQE expression vector *E. coli* strain M15[pREP4] can be used for the production of recombinant proteins.

3.1.3 Mammalian Expression Vector pRc-RSV

The pRc-RSV plasmid is an eukaryotic expression vector that contains enhancer-promoter sequence from the Rous sarcoma virus long terminal repeat for high level transcription; polyadenylation signal and transcription termination sequences from the bovine growth hormone to enhance RNA stability and the neomycin or geneticin resistance gene for selection (Yamamoto *et al*., 1980, Gorman *et al*., 1982) .

3.1.4 *E. coli* Vector pQE-60

pQE-60 (Qiagen, Hilden, Germany) belongs to the pDS family of plasmids (Bujard *et al*., 1987) and contains a T5 promoter, a 6xHis-tag coding sequence and the β-lactamase gene that confers resistance to ampicillin.

3.1.5 Chemicals

Most of the chemicals used were obtained from Sigma, Munich, Germany or from Merck, Darmstadt, Germany.

3.2 Methods

3.2.1 Generation of Expression Vectors Encoding Bi- and Trifunctional Inhibitors

Expression vectors encoding bi- and trifunctional inhibitors were generated by Dr. Ulla Magdolen (Clinical research group, Frauenklinik der TU München). The cDNAs encoding human TIMP-1, human TIMP-3 (both kindly provided by A. Krüger, Institut für Experimentelle Onkologie und Therapieforschung, TU München, Germany), as well as chicken cystatin (chCysWT; Muehlenweg *et al.,* 2000) were used as templates for multiple PCR amplification steps to generate genes encoding bifunctional inhibitors. For synthesis of genes encoding trifunctional inhibitors, the cDNA encoding chCys-uPA$_{19-31}$ was used, a cystatin variant, that harbors the uPAR-binding site of the uPA molecule (Muehlenweg *et al.,* 2000).

The coding regions of the N-terminal domains of TIMP-1 (aa 1-128) and TIMP-3 (aa 1-123) were fused to the coding regions of chCysWT (aa 1-115) and of chCys-uPA$_{19-31}$ consisting of 117 aa. Between the two inhibitor domains, a stretch of 10 aa was introduced as a flexible linker. The constructs were inserted into the multiple cloning site of the *E. coli* expression vector pQE-60 which provides a C-terminal (histidine)$_6$-tag. The recombinant plasmids were transformed into *E. coli* strain M15 (pREP4).

For generation of eukaryotic expression plasmids, a two-step cloning strategy was applied: (i) pQE-60-derived plasmids encoding the various bi- and trifunctional inhibitors were used as templates for PCR amplification of the cystatin-encoding part including the N-terminally located flexible linker and the C-terminal (histidine)$_6$-tag. These fragments were directionally subcloned into the eukaryotic expression vector pRc-RSV. ii) The coding regions of the N-terminal domains of TIMP-1 (aa 1-128) *plus* its N-terminally located signal sequence consisting of 23 aa and TIMP-3 (aa 1-123) *plus* its native signal sequence (23 aa), respectively, were amplified and inserted 5' of the cystatin variants-encoding sequences. The deduced aa sequences of the mature recombinant fusion proteins (expressed by eukaryotic cells) are identical to those of the proteins expressed in *E. coli*. In all cases, in the expression

vectors the correct orientation and sequence of the cloned DNA was verified by sequencing (TopLab, Martinsried, Germany).

3.2.2 Bacterial Expression of the Multifunctional Inhibitors

E. coli strain M15[pREP4] clones transformed with recombinant inhibitors were grown in 10 ml of LB medium containing 100 mg/ml ampicillin (Sigma, St. Louis, Mo, USA) and 25µg/ml kanamycin (Sigma, St. Louis, Mo, USA)on an orbital shaker at 200 rpm., at 37˚C overnight. Then, 5 ml of the overnight cultures were inoculated in 200 ml of prewarmed LB medium supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin and grown at 37˚C with vigorous shaking until an OD_{600} of 0.8-0.9. The expression of the recombinant protein was induced for 5 h by addition of IPTG in a final concentration of 2 mM.

LB medium   10 g/l trypton (Difco, Detroit, MI, USA)
                     5 g/l yeast extract (Difco, Detroit, MI, USA)
                     10 g/l NaCl

3.2.3 Purification of the Multifunctional Inhibitors

Purification of the proteins was performed using Ni^{2+}-NTA affinity chromatography. This method is based on the interaction of the recombinantly introduced 6xHis-tag into the protein of interest with a nickel ion of a tetradentate chelating adsorbent.

Purification of recombinant inhibitors was performed under denaturing and slightly reducing conditions. The bacterial cells were harvested by centrifugation (4000 x g, RT, 20 min) and then lysed in buffer A by gently shaking for 2 h at RT. The cell debris was removed by centrifugation (10000 x g, RT, 25 min) and the supernatant subjected to Ni^{2+}-NTA-agarose (QIAgen, Hilden, Germany) affinity chromatography. After four washing steps, first with 20 ml buffer A, second with 40 ml buffer B and, subsequently, with 20 ml buffer C and D, the proteins were eluted with 10 ml of buffer E.

Buffer A   6 M GuHCl
                     100 mM NaH_2PO_4
                     10 mM Tris-HCl, pH 8.0
                     8 mM β-mercaptoethanol

Buffer B   8 M urea
                            100 mM NaH$_2$PO$_4$
                            100 mM Tris-HCl, pH 8.0
                            8 mM β-mercaptoethanol

Buffer C   buffer B, pH 7.0

Buffer D   buffer B, pH 6.3

Buffer E   buffer B, pH 4.1

3.2.4 Refolding of the Multifunctional Inhibitors

For refolding of N-hTIMP-1-chCysWT and N-hTIMP-1-chCys-uPA$_{19-31}$, DTT was added at a final concentration of 50 mM and the proteins were dialyzed against dialysis buffer I for 8 h at RT, followed by two dialysis steps at 4 °C each for 48 h against dialysis buffer II and one dialysis step at 4 °C against PBS, pH 7.4, for 24 h.

For refolding of N-hTIMP-3-chCysWT and N-hTIMP-3-chCys-uPA$_{19-31}$, a modification of the method described by Negro *et al.* (1997) was used. For this 2 ml of Ni^{2+}-NTA resin and β-mercaptoethanol in a final concentration of 8 mM were added to 1 mg of eluted protein. The protein solution was gently shaken for 2 h at RT and then dialyzed against 100 mM sodium phosphate, pH 8.3, for 72 h at 4 °C. The refolded protein was eluted from the resin with 8 M urea, 100 mM NaH$_2$PO$_4$, 10 mM Tris-HCl, pH 4.1, and then dialyzed against PBS, pH 7.4 for 24 h. Refolded proteins were stored in aliquots at –20 °C until use.

Dialyse buffer I   6 M urea
                            0.02 % NaN$_3$
                            50 mM Tris-HCl, pH 8.0

Dialyse buffer II   2 M urea
                            300 mM NaCl
                            2.5 mM reduced glutathione
                            0.5 mM oxidized glutathione
                            0.02 % NaN$_3$
                            50 mM Tris-HCl, pH 8.0

3.2.5 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

The SDS-PAGE (Laemmli) buffer system is a discontinuous buffer system that incorporates SDS in the buffer. By using different buffers in the gel and in the electrode solutions and adding a stacking gel to the resolving gel, samples are compressed into a thin starting band and individual proteins are finely resolved and separated. In this system, proteins are denatured by heating in buffer containing SDS and a thiol reducing agent such as β-mercaptoethanol. The resultant polypeptides take on a rod-like shape and a negative charge and migrate to the anode according to their molecular weight.

To prove the expression of recombinant inhibitors by E. coli, to determine their purity after Ni^{2+}-NTA-agarose affinity purification and to estimate the amount of proteins after refolding, 12% SDS polyacrylamide minigel was used. Prior to loading into the gel the probes were mixed 1: 2 with SDS reducing sample buffer and heated at 95 °C for 3 min. To facilitate the identification of the bands, a SDS-PAGE protein standard [Low Range (BIO-RAD, Krefeld, Germany)] was loaded on to the same gel. The electrophoresis was performed in running buffer under the constant voltage of 150 V. After electrophoresis, the gel was fixed for 30 min, stained with Coomassie R-250 for 1 h and destained with 10% acetic acid for 2 h.

SDS-Polyacrylamide Resolving Gel Buffer (10 ml)

	12 %	15 %
DDI H_2O (ml)	4.35	3.4
40 % acrylamide/bis (ml)	3.0	3.75
1.5 M Tris-HCl, pH 8.8 (ml)	2.5	2.5
10 % SDS (ml)	0.1	0.1
10 % APS* (μl)	80	80
TEMED* (μl)	10	10

*added immediately prior to pouring the gel

SDS-Polyacrylamide stacking gel buffer (10 ml)   6.4 ml DDI H_2O
    1 ml 40 % acrylamide/bis
    2.5 ml 0.5 M Tris-HCl, pH 6.8
    0.1 ml 10 % SDS
    80 μl 10 % APS*
    10 μl TEMED*

*added immediately prior to pouring the gel

Running buffer, pH 8.3 (1l)   14.4 g glycine
                                           3.0 g Tris base
                                           1.0 g SDS

SDS-PAGE reducing bample buffer   3.55 ml deionized water
                                           1.25 ml 0.5 M Tris-HCl, pH 6.8
                                           2.5 ml glycerol
                                           2.0 ml 10% SDS
                                           0.2 ml 0.5%(w/v) bromophenol blue
                                           0.5 ml β-mercaptoethanol

SDS-PAGE non-reducing sample buffer   SDS sample buffer without β-
                                           mercaptoethanol

Fixation solution   40 % ethanol
                                           10 % acetic acid

Staining solution   0.1 % Coomassie R-250
                                           10 % acetic acid

3.2.6 Reverse Zymographic Analyses

The inhibitory activity of recombinant trifunctional inhibitors against the cysteine protease papain was tested by reverse zymography. A 15 % SDS polyacrylamide minigel containing 0.1 % (w/v) casein (Sigma, Munich, Germany) and a control gel without casein were prepared. The inhibitor samples (≈ 0.25 µg) were loaded in nonreducing sample buffer into the wells of these gels and electrophoresis was performed. After electrophoresis, gels were washed in 2.5 % Triton X-100 three times for 30 min at RT to remove SDS and then incubated in 100 mM phosphate buffer, pH 6.0, containing 2 mM cysteine, 1 mM EDTA, and 0.01 mg/ml papain (40 °C for 2.5 h). Afterwards, the gels were stained with 0.05 % Coomassie blue in 40 % ethanol/10 % acetic acid solution overnight and destained in 35 % ethanol/10 % acetic acid for 3 h.

The inhibitory activity of recombinant bi- and trifunctional inhibitors against MMP-2 was also tested by reverse zymography. Minigels were prepared containing 12.5 % SDS polyacrylamide, 1 mg/ml gelatin (Sigma, Germany), 0.5 µg/ml MMP-2 (Roche, Manheim, Germany). The inhibitor samples (≈ 0.25 µg) each in nonreducing sample buffer were loaded and electrophoresis was performed at 15 mA at 4 °C. After electrophoresis, the gels were washed in 50 mM Tris-HCl, pH 7.5, 5 mM CaCl$_2$, 5 µM ZnCl$_2$, 2.5 % Triton X-100 for 3 h, at 37 °C with three changes of buffer. Incubation was performed in 50 mM Tris/HCl, pH 7.5, 5

mM $CaCl_2$, 5 μM $ZnCl_2$, 0.02 % NaN_3 at 37 $^{\circ}$C overnight. After fixation in 40 % ethanol/10 % acetic acid for 10 min, the gels were stained with 0.5 % Coomassie blue in 35 % ethanol/10 % acetic acid for at least 2 h and destained in 35 % ethanol/10 % acetic acid. A control gel without gelatin and without MMP-2 was run and stained as well.

3.2.7 Determination of K_i-Value Towards Papain and Active Concentrations of Multifunctional Inhibitors

The determination of the inhibitory activity of the multifunctional inhibitors towards the cysteine protease papain using continuous fluorimetric inhibition assays was performed in the cooperation with Prof. Dr. W. Machleidt and Dr. I. Assfalg-Machleidt from the Adolf-Butenandt Institute für Physiologische Chemie, LMU München.

Inhibition of papain (10 pM, Roche Molecular Biochemicals) was assayed at 30 °C with the fluorogenic substrate Z-Phe-Arg-NHMec (Bachem, Heidelberg, Germany) in kinetic buffer. The inhibition constants (Ki) for papain were calculated from the rate constants ($K_i = k_{off}/k_{on}$), which were obtained from pre-steady-state analysis.

Inhibitory active concentrations of recombinant inhibitors were determined by titration with E-64 standardized papain (4 nm) using the 10 μm substrate Z-Phe-Arg-NHMec (Machleidt *et al.*, 1993; Barrett and Kirschke, 1981).

Kinetic buffer   250 mM sodium acetate buffer, pH 5.5
                             2 mM EDTA
                             0.015 % BRIJ^{TM} 35
                             1 mM DTT
                             1 % DMSO

3.2.8 MMP Activity Assays

The inhibitory activity of the recombinant bi- or trifunctional inhibitors against matrix metalloproteases was tested applying MMP-1, -2, and -9 activity assays (Biotrak^{TM}; Amersham Biosciences, Freiburg, Germany). 50 μl of enzyme (MMP-2: 6 ng/ml, MMP-1: 25 ng/ml, MMP-9: 8 ng/ml) were given into each well of the microtiter plate, coated with different anti-MMP antibodies for the various MMP-assays. Afterwards MMPs were activated by adding 0.5 mM APMA (50 μl/well) and incubating the plate for 15 min at 37 $^{\circ}$C. Then, inhibitors (0.5-2 μg in 10 to 40 μl) were added and incubated for 20 min at RT, and, finally,

the microtiter plate of the various MMP-assays was incubated overnight at 4 °C. As a negative control, a mixture without MMPs was used; as positive controls, MMPs were incubated with PBS instead of inhibitors, or heat-denatured (10 min at 95 °C) inhibitors were used. The next day, the plates were washed four times with wash buffer and 100 μl of detection reagent (consisting of detection enzyme and chromogenic substrate) were added to each well and the resulting color changes were monitored over several hours in a spectrophotometer at 405 nm.

Assay buffer	50 mM Tris-HCl, pH 7.6
	1.5 mM NaCl
	0.5 mM $CaCl_2$
	1 μM $ZnCl_2$
	0.01 % (v/v) BRIJ[TM] 35
	0.01 % (w/v) gelatin (only in the MMP-2 assay)
	500 μg/ml BSA
Wash buffer	10 mM sodium phosphate, pH 7.0
	0.05 % Tween[TM] 20

3.2.9 Flow Cytofluorometry (FACS)

The binding of the N-TIMP-1/cystatin variant containing the u-PAR-directed binding epitope of u-PA to cell surface-associated uPAR was proved in FACS analysis. 5×10^6 U937 cells were stimulated with 1 mM (final concentration) of PMA (Sigma-Aldrich, Taufkirchen, Germany) for 72 h at 37 °C. After washing with PBS, the cells were incubated with 50 mM glycine/100 mM NaCl, pH 3.0, for 1 min at RT to dissociate endogenous receptor-bound uPA and then the acidic buffer was neutralized with an equal volume of 500 mM HEPES/100 mM NaOH, pH 7.5. After washing, the cells were resuspended in PBS/0.1 % BSA (107 cells/ml) and then 2.5×10^5 cells were incubated for 30 min with a mixture of FITC-labeled HMW-uPA (16 ng) *plus* different concentrations of refolded N-hTIMP-1-chCysWT, or N-hTIMP-1-chCys-uPA$_{19-31,}$ or the amino terminal fragment of uPA. Prior detection of cell-associated fluorescence propidium-iodid was added to identify died cells and exclud them from evaluation.

3.2.10 Surface Plasmon Resonance Analysis

Biomolecular Interaction Analysis (Real-time BIA) uses the optical phenomenon surface plasmon resonance (SPR) to investigate interactions between biomolecules at the surface of a sensor chip. One of the components tested for interaction is immobilized on the surface and the other flows over the surface in free solution. As the interaction proceeds, the concentration of analyte in the surface layer changes, giving an SPR response, which can be followed in real-time.

Real-time BIA technology was used to analyze the simultaneous interaction of the recombinant inhibitor proteins with the cysteine protease papain and MMP-2 utilizing the BIAcore 2000 system (BIAcore AB, Uppsala, Sweden). All experiments were performed at 25 °C at a flow rate of 5 µl/min. Papain (Roche, Manheim, Germany) was immobilized to a CM-5 (research grade, Biacore AB, Uppsala, Sweden) chip using the amino-coupling kit according to the manufacturer's recommendation. For this, the dextran matrix of the chip was activated with 50 mM NHS (Biacore AB, Uppsala, Sweden) und 200 mM EDC (Biacore AB, Uppsala, Sweden), followed by an injection of papain (48 µg/ml in 100 mM acetic acid, pH-6). Remained active ester groups were deactivated with 1 M ethanolamine, pH 8.5 (Biacore AB, Uppsala, Sweden). Then, 200 µl of N-hTIMP-1-chCysWT or N-hTIMP-1-chCys-uPA$_{19-31}$ or chCysWT (final concentration 50 µg/ml) in HBS-P-BSA buffer were applied to the papain-coupled chip to form a papain-cystatin complex. After intensive washing with HBSP buffer at a flow rate of 50 µl/min, 110 µl of different concentrations (51, 68, and 102 µg/ml) of MMP-2 (Roche, Manheim, Germany), activated by APMA (30 min at 37 °C), were injected to follow the formation of the ternary papain-[N-hTIMP-1-cystatin(variant)]-MMP-2 complex. Regeneration was achieved by injection of HBS-EP puffer. The association and dissociation constants of the interaction of MMP-2 with the N-hTIMP-1-chCys-uPA$_{19-31}$ and N-hTIMP-1-chCysWT were determined using the BIA evaluation software of BIAcore.

HBS-P-BSA buffer	10 mM HEPES, pH 7.4
	150 mM NaCl
	0.005 % (v/v) Tween 20
	0.05 % BSA
HBS-EP	10 mM HEPES, pH 7.4
	150 mM NaCl
	0.005 % (v/v) Tween 20
	3 mM EDTA

3.2.11 Generation of a Polyclonal Antibody Directed Against chCys

Polyclonal antibodies directed against chCys were generated in cooperation with Dr. Pineda (Pineda Antikörperservice, Berlin, Germany). chCysWT, that served as antigen for immunization, was expressed in *E. coli* as described in 3.2.2. Purification of the proteins was performed using Ni^{2+}-NTA affinity chromatography (s. 3.2.3) followed by refolding procedure as described for N-hTIMP-1-chCysWT and N-hTIMP-1-chCys-uPA$_{19-31}$. Approximately 200 µg of the refolded chCysWT were mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously to two rabbits. Two booster injections were administered 4 and 8 weeks later. Rabbits were bled on the day 61, 100 and 130 from the initial immunization and blood was allowed to stand for 4 h at RT and 4 °C overnight. After removal of clot and debris by centrifuging 20 min at 3000g, the sera were tested for the presence of a specific antibody by immunoblotting.

3.2.12 Western Blott Analysis

To test the reactivity of polyclonal antibodies directed again chCys in sera from immunized rabbits, semi-dry Western blott analyses were performed. About 1 µg of different proteins serving as positive or negative controls were mixed with the equal volume of SDS-containing reducing or non-reducing sample buffer and loaded into the wells of a 12 % SDS-polyacrylamid gel. As a standard, BenchMark^{TM} Prestained Protein Ladder (Gibco, Karlsruhe, Germany) was used. After electrophoresis the gel was incubated for 5 min in blotting solution and a "sandwich" consisting of three layers of Whatman filter paper soaked with 20% methanol in 50 mM boric acid pH 9.0, the PVDF-membrane, the gel, three layers of Whatman paper soaked in blotting solution was constructed. The blotting was performed using Biometra transfer device (Biomedizinische Analytik, Göttingen, Germany) at 50 V, maximum 4 mA/cm^2 for 2 h. Afterwards, the membrane was blocked in PBS/5 % skim milk powder (Merck, Darmstadt, Germany) for 1 h at RT and then incubated with anti-chCysWT polyclonal antibody diluted 1 : 2000 in PBS/1 % skim milk powder at 4 °C overnight. After washing for four times with PBS/1 % skim milk powder the membrane was incubated with the peroxidase labeled secondary goat anti-rabbit polyclonal antibodies (Dianova, Germany), diluted 1: 5000 in PBS/1 % skim milk powder, again washed and finally the antigen-antibody reaction was visualized using the "ECL Western Blotting Detection Reagent" (Amersham Pharmacia, Freiburg, Germany) according to the manufacturer's manual.

Blotting solution   5 % methanol
                                            50 mM boric acid, pH 9.0.

3.2.13 Determination of Total Protein Concentration

Concentration of chCysWT that served as a standard in chCys-ELISA was determined using the BCA Protein Quantitation Kit (Pierce, Rockford, IL, USA) according to the manufacturer's manual. This method is based on reduction of Cu^{+2} by protein to Cu^{+1} in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) by a reagent containing bicinchoninic acid (BCA). For this assay several dilutions of chCysWT in TBST (TBS + 0.1 % Triton X-100 + 0.05 % Tween 20)were prepared, incubated with BCA Protein Assay Reagent on a microtitre plate and the resulting color reaction was measured at a wavelength of 540 nm.

3.2.14 Cell Culture

OV-MZ-6#8 cells are adherent cells and propagate as a monolayer attached to the culture vessel. They were cultured under standard conditions (37 °C, 5 % CO_2, humid atmosphere) in DMEM, containing 10 mM HEPES, 10% FCS, streptomycin/penicillin (100 µg/ml, 100 U/ml) (all from Gibco, Karlsruhe, Germany) and 0.27 mM asparagine, 0.55 mM arginine (both from Sigma, St. Louis, Mo, USA). Cells were subcultured 3 times a week. For experiments were used 70-80 % confluent cells between passage 2 to 9. Every 6 weeks the cells were tested for mycoplasma contamination by PCR.

U937 cells are suspension cells, and were cultured under standard conditions in RPMI medium, containing 10 mM HEPES, 2mM L-glutamin, 10% FCS, and streptomycin/penicillin (100 µg/ml, 100 U/ml) (all from Gibco, Karlsruhe, Germany). The cells were maintained in culture by transfer 1 ml of cell suspension in 30 ml cell culture medium once per week. For FACS experiments, the cells were stimulated with PMA, which made cells adherent and led to a 10-fold over expression of uPAR on the cell surface.

3.2.15 Stable Transfection of OV-MZ-6#8 Cells

For transfection, OV-MZ-6#8 cells were grown in 6-well plate until 40-60 % confluence was reached. 15 µl of SuperFect™ (QIAgen, Hilden, Germany) and 2 µg of the various eukaryotic expression plasmids (pRc-RSV-N-hTIMP-1-chCysWT; pRc-RSV-N-hTIMP-1-chCys-uPA$_{19-31}$; pRc-RSV-N-hTIMP-3-chCysWT; pRc-RSV-N-hTIMP-3-chCys-uPA$_{19-31,}$ and empty vector pRc-RSV) were dissolved in 100µl DMEM (without serum and antibiotics), thoroughly mixed and incubated for 15 min at RT to allow transfection-complex formation. Then, 400 µl FCS-free cell culture medium were added to the SuperFect-DNA mixture and transferred to the cells that were previously washed with PBS (Gibco, Karlsruhe, Germany). After 4 h of incubation at 37 °C cells were washed for 3 times with PBS and further cultured in 10 % FCS DMEM. At 80-90 % confluency, the selection of stably transfected cells using cell culture medium containing 1 µg/ml G 418 (Gibco, Karlsruhe, Germany) was started. Selected cell were seeded into the wells of a 24-well plate (30, 000 cells in 1 ml 10% FCS DMEM) and incubated for 48 h. After incubation, the cell culture supernatants were collected and frozen at –20 °C until used for detection of multifunctional inhibitors expression by a newly developed ELISA.

3.2.16 Cell Proliferation Assay

The proliferation assay was performed to compare the growth rate of vector transfected cells and cells, transfected with the expression plasmids encoding multifunctional inhibitors.
For this, 30,000 cells in 1ml 10 % FCS DMEM were seeded in triplicate into the wells of an 24-well plate and incubated for 48 or 96 hours. After incubation cells were washed with PBS, detached from the plate by incubation with 1 % (w/v) EDTA/PBS solution and counted in Neubauer hemocytometer upon Trypan blue exclusion.

3.2.17 Cell Adhesion Assay

Fibronectin (Becton Dickinson, Heidelberg, Germany), vitronectin (Promega, Germany), and collagen type IV (Sigma, München, Germany) were diluted with PBS to the final concentration of 10 µg/ml. For coating, 100 µl of the protein solution were added to each well of a 96-well plate and incubated overnight at 4 °C. The next day, wells were washed two times with PBS, blocked with PBS/2 % BSA for 3 h at RT and again washed with PBS.

Afterwards 40,000 cell in 100 µl DMEM/0.5 % BSA were seeded to each well, the plate was incubated for 2 h at 37 ^{o}C and washed carefully with PBS to remove nonadherent cells. The adherent cells were visualized by incubating them with substrate solution (50 µl/well + 50 µl/well PBS) for 1 h at 37 ^{o}C. The resulting color reaction was stopped with the stop solution and the absorbance measured in a microplate reader at 405 nm.

The serial dilutions of cell suspension in a range from 40,000 to 2,500 cells in 50 µl PBS served as a standard.

Substrate solution   15 mM p-nitrophenol-N-acetyl-beta-D-glucosaminid
                                              0.5 % Triton X-100
                                              100 mM sodium acetate, pH 5.0

Stop solution   200 mM NaOH
                                                5 mM EDTA

3.2.18 Cell Invasion Assays

Invasion assays were performed according to Szpaderska and Frankfater (2001). Aliquots of Matrigel (11.3 mg/ml, Becton Dickinson Labware, Bedford, MA) were stored frozen at –20 ^{o}C. After thawing on ice overnight the Matrigel was diluted 1:24 with cold PBS and the membrane inserts (6.4 mm diameter, 8 µm pores, Becton Dickinson Labware, Bedford, MA) for 24 well plates were coated with 60 µl Matrigel per insert (density of 85 µg/cm^{2}). The plates were incubated for 3 h at 37 ^{o}C in a cell culture incubator. After gelling the Matrigel was dried overnight in uncovered plates in a laminar hood. The next day, the gel was rehydrated for 2 h by addition of 200 µl serum-free DMEM/0.1 % BSA. Transfected cells were grown until 60 to 80 % confluency and adjusted to 10^{5} cells/ml DMEM/0.1 % BSA. 5 x 10^{4} transfected cells/500 µl medium were seeded into each insert. The lower chambers of the inserts were filled with 750 µl DMEM containing 10 % FCS as a chemoattractant. For every value, assays were performed in triplicate. After 48 h of incubation the Matrigel with the noninvaded cells was wiped off with a tissue and invaded cells on the lower side of the filter were fixed and stained (Diff-Quick®, Dade Behring AG, Switzerland). The stained cells were counted under the microscope (100 x magnification) with the help of a grid.

Likewise, cell invasion assays with recombinant inhibitors isolated from *E. coli* were performed. Wild type OV-MZ-6# 8 cells (5x10^{4} cells) in 400 µl DMEM were applied to each insert and recombinant inhibitors (up to 50 µg inhibitor protein in 150 µl) were added to the

cells together with 500 µg BSA. Controls received PBS with 500 µg BSA. As another control, heat-denatured inhibitors (10 min at 95 ^{o}C) were used. The invasion was stopped after 24 h of incubation and the level of cell invasion was detected as described above.

3.2.19 Tumor Model

Pathogen-free, female athymic (nu/nu, CD1) mice (9 weeks old), were obtained from Charles River Laboratories (Sulzfeld, Germany). The animal experiments were performed in cooperation with PD. Dr. A. Krüger, Institut für Experimentelle Onkologie und Therapieforschung, TU München, Germany

Stably transfected cells were grown until 60-80 % confluency, detached from the culture flasks and adjusted to 14 x 10^6 cells/ml PBS. Then 500 µl of the cell suspension (7 x 10^6 cells) were inoculated into the peritoneal cavity of nude mice. After 56 days mice were sacrificed, all intraperitoneal organs, including the tumor, removed and weighed. These data were taken further as total situs weight. Then all visible tumor mass was removed and subsequently weighed. To estimate the differences in tumor between individual mice the ratio of tumor mass to the weight of the total situs was calculated. Tumors of three mice from each group were frozen in liquid nitrogen for further detection of the multifunctional inhibitors antigen content by ELISA. Blood and ascitic fluid were collected, centrifuged at 2000 x g, 30 min at 4 ^{o}C and stored at -20 ^{o}C for further detection of the antigen level of multifunctional inhibitors.

3.2.20 Preparation of Tumor-Tissue Extracts

For detection of the antigen level of multifunctional inhibitors in tumor tissues, tumors were pulverised using Dismembrator (Braun, Melsungen). The powder was suspended in TBS/1% (v/v) Triton X-100, incubated overnight at 4 ^{o}C under rotation and centrifuged at 13,000 x g, for 60 min, at 4 ^{o}C. Supernatants were subjected to ELISA.

3.2.21 Statistical Data Analysis

Differences in tumor weight over total situs weight between the groups were calculated using the Man-Whitney Rank Sum Test owing to non normal distribution of the data. A significance level of $p<0.05$ was considered statistically significant.

4. Results

4.1 Expression, Purification and Refolding of Multifunctional Inhibitors

Bifunctional inhibitor molecules were designed which are composed of the inhibitory domains of human TIMP-1 or TIMP-3 (N-hTIMP-1 or -3) fused to chicken wild-type cystatin (chCysWT) and thus, are directed against both matrix metalloproteinases and cysteine proteases. Additionally, trifunctional inhibitors were generated, in which a cystatin variant was used that harbors the uPAR-binding site of the uPA molecule (uPA_{19-31}) and blocks uPA/uPAR-interaction. As an example, the structure of the trifunctional inhibitor N-hTIMP-1-chCys-uPA_{19-31} is depicted in Figure 11.
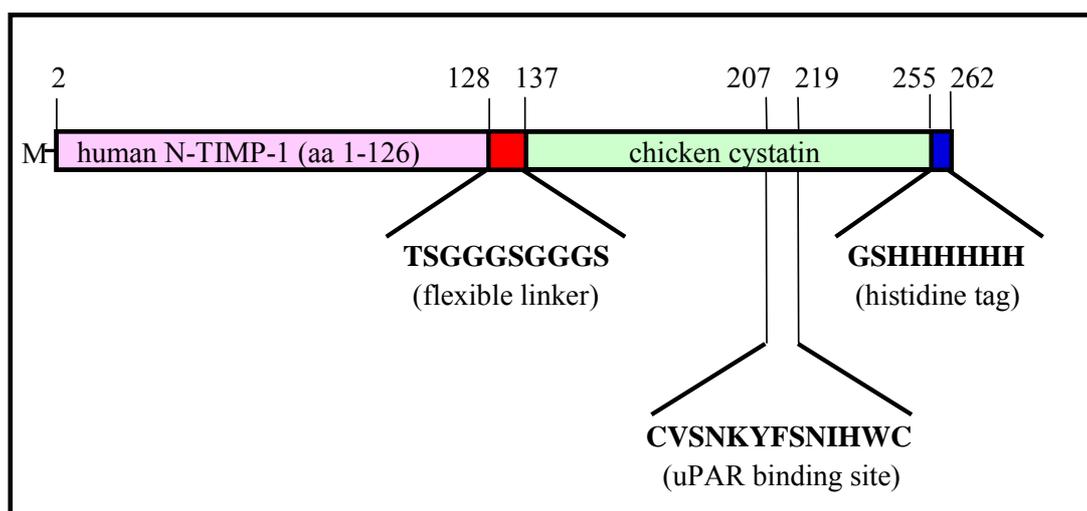

Figure 11. *Desing of the trifunctional inhibitor N-hTIMP1-chCys-uPA$_{19-31}$.* The cDNA sequence encoding the N-terminal domain of human TIMP-1 was recombinantly fused to the DNA sequence encoding chCys-uPA_{19-31}. Between the two inhibitor domains, a stretch of 10 aa was introduced as a flexible linker. The construct was cloned into the bacterial expression vector pQE-60 which provides a C-terminal (histidine)$_6$-tag. In a similar manner, the fusion genes encoding N-hTIMP-1-chCysWT, N-hTIMP-3-chCysWT, and N-hTIMP-3-chCys-uPA_{19-31} were designed and generated (all constructs were established in cooperation with Dr. U. Magdolen, Clinical Research unit, Frauenklinik der TU München).

All four recombinant inhibitors (N-hTIMP-1-chCysWT, N-hTIMP-3-chCysWT, N-hTIMP-1-chCys-uPA$_{19-31}$, and N-hTIMP-3-chCys-uPA$_{19-31}$) were expressed in *E. coli* with an expression rate of $\approx$ 10-15 % of total protein (Fig. 12A). The introduction of a (histidine)$_6$-tag at the C-terminus allowed purification of the recombinant proteins by use of Ni^{2+}-nitrilotriacetic acid affinity chromatography, yielding a purity of > 95 % under denaturing and reducing conditions (Fig. 12B). The inhibitors contain structural and functional important intramolecular disulfide bridges; therefore, the purified proteins had to be refolded to obtain biologically active proteins. The protocol used for refolding of N-hTIMP-1-chCysWT and N-hTIMP-1-chCys-uPA$_{19-31}$ was not suitable for refolding of N-hTIMP-3-containing recombinant proteins (more than 90 % of the protein precipitated). After testing different protocols for refolding of the N-hTIMP-3 variants, a modification of the method described by Negro *et al.* (1997) was used instead. The highest achievable active concentration of purified recombinant proteins corresponded to only about 8 % of total protein. The reason for the low recovery of active inhibitors is very likely due to the presence of six disulfide bridges in all four recombinant inhibitors (three in the N-terminal domain of TIMP-1/-3 and three in chCys), that have to be correctly formed during the refolding process (Magdolen *et al.*, 2002).

A

B
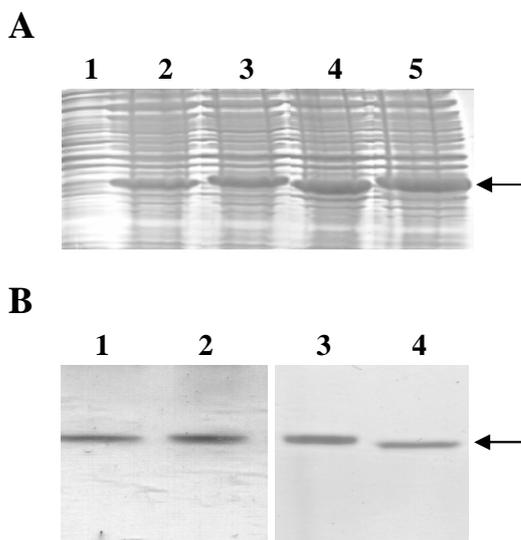
Figure. 12. *Expression and purification of recombinant inhibitors.* **A**. Bacterial lysates before (line 1) and after induction of protein expression (lines 2-5) were subjected to SDS-PAGE analyses and subsequently stained with Coomassie blue. 1: cell extract from recipient *E. coli* strain; 2: N-hTIMP-1-chCysWT; 3: N-hTIMP-1-chCys-uPA$_{19-31}$; 4: N-hTIMP-3-chCysWT; 5: N-hTIMP-3-chCys-uPA$_{19-31}$.
B. SDS-PAGE analysis of multifunctional inhibitors purified by Ni^{2+}-NTA affinity. 1: N-hTIMP-1-chCysWT; 2: N-hTIMP-1-chCys-uPA$_{19-31}$; 3: N-hTIMP-3-chCysWT; 4: N-hTIMP-3-chCys-uPA$_{19-31}$.

4.2 Inhibition of the Cysteine Protease Papain

The inhibitory activity of recombinant multifunctional inhibitors towards the cysteine protease papain was tested by reverse zymography (Fig. 13). About 0.25 µg of purified and refolded multifunctional inhibitors as well as recombinant chCysWT were subjected to a 0.1 % casein-containing SDS polyacrylamide gel electrophoresis. After removal of SDS, the gel was incubated in a buffer containing papain, added to digest its substrate, casein. The bands on the Coomassie-stained gel correspond to non-degraded casein at the positions of chCysWT ($\approx$ 13 kDa) and multifunctional proteins ($\approx$ 31 kDa) (Fig. 13A). A control gel lacking casein was treated in the same way and showed no bands (Fig. 13B).
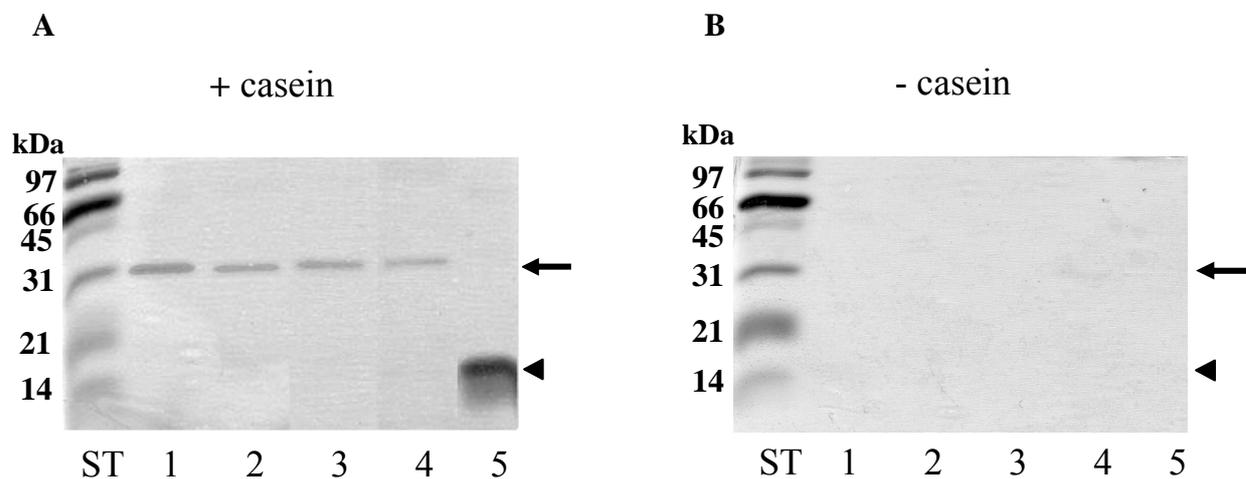

Figure 13. *Reverse zymography to analyze the inhibitory activity towards papain.* **A.** About 0.25 µg of recombinant N-hTIMP-1-chCysWT (lane 1), N-hTIMP-3-chCysWT (lane 2), N-hTIMP-1-chCys-uPA$_{19-31}$ (lane 3), N-hTIMP-3-chCys-uPA$_{19-31}$ (lane 4), and chCysWT (lane 5), respectively, were loaded into wells of an SDS polyacrylamide gel containing casein. After electrophoresis, the gel was washed extensively to remove SDS and incubated in a solution containing papain for the digestion of the casein substrate. Subsequently, the gel was stained with Coomassie blue. **B.** Control gel lacking casein.

In addition, the K_i-values of the recombinant proteins towards papain were determined (Table 1). Recombinant N-hTIMP-1-chCysWT, N-hTIMP-3-chCysWT, and N-hTIMP-1-chCys-uPA$_{19-31}$, display K_i-values close to that of natural chicken cystatin from egg white, indicating that insertion of the uPA-derived peptide (uPA$_{19-31}$) as well as the presence of the N-terminal domain of TIMP-1/-3 and the C-terminal (histidine)$_6$-tag have no negative influence on inhibitory activity towards cysteine proteases.

Table 1. *Inhibition constants of multifunctional inhibitors towards papain*

Inhibitor	K_i [pM]
Natural chCys[a]	1.4
chCysWT[b]	2.0
chCys-uPA$_{19-31}$[b]	1.9
N-hTIMP-1-chCysWT	2.0
N-hTIMP-1-chCys-uPA$_{19-31}$	1.9
N-hTIMP-3-chCysWT	2.0
N-hTIMP-3-chCys-uPA$_{19-31}$[c]	n.d.

[a] The inhibition constant for natural chCys was taken from Machleidt *et al.* (1993).

[b] The inhibition constants for recombinant chCysWT and chCys-uPA$_{19-31}$, respectively, were taken from Muehlenweg *et al.* (2000).

[c] The inhibition constant of N-hTIMP-3-chCys-uPA$_{19-31}$ could not be determined, because of a too low active concentration of this inhibitor after refolding

4.3 Inhibition of Matrix Metalloproteinases by Recombinant Inhibitors

The inhibitory capacity of the TIMP-domains of the bi- and trifunctional inhibitors was tested in MMP activity assays. The multifunctional inhibitors as well as recombinantly expressed, purified and refolded N-hTIMP-1 significantly reduced the proteolytic activity of MMP-2 (Fig. 14). With heat-denatured inhibitors added to the wells as negative control samples, inhibition was almost but not completely abolished. The residual inhibitory activity may be due to renaturation of part of the inhibitors after heat-denaturation. All multifunctional inhibitors and N-hTIMP-1 were additionally analyzed in MMP-1 and MMP-9 assays and were shown to reduce the activity of both matrix metalloproteinases by about 40-90 % (data not shown).
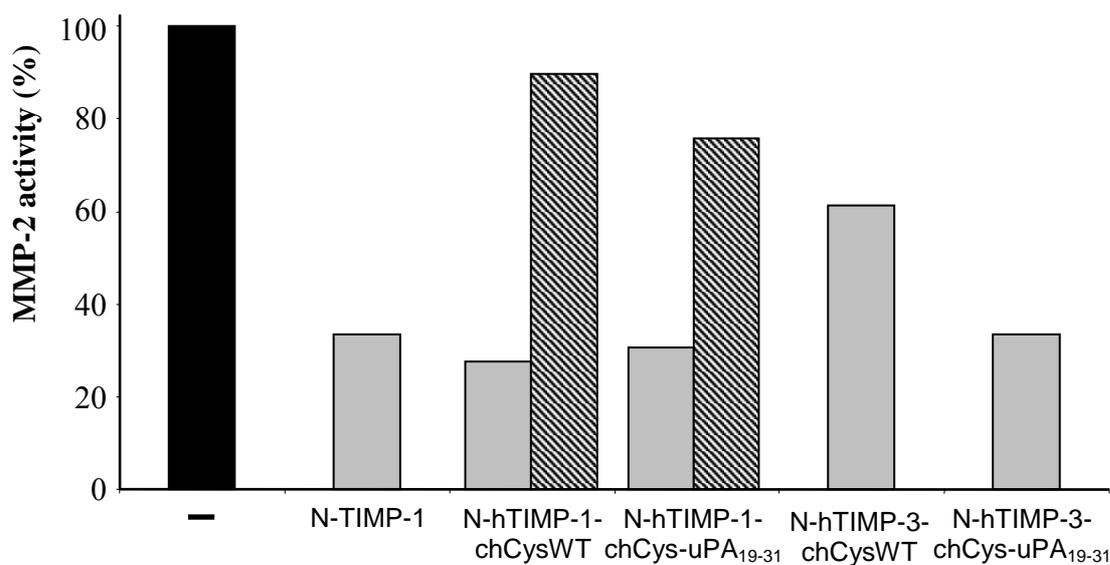

Figure 14. *Activity of recombinant inhibitors as tested by the Biotrak MMP-2 activity assay.*
0.5 µg to about 2 µg of recombinant inhibitors were incubated with activated MMP-2 (6 ng/ml) overnight at 4 °C. Then, the detection enzyme and chromogenic substrate were added and the residual MMP-2 activity was monitored in a spectrophotometer at 405 nm. As a control, N-hTIMP-1-chCysWT and N-hTIMP-1-chCys-uPA$_{19-31}$ were inactivated at 96 °C for 10 min prior to incubation with activated MMP-2 (hatched boxes). The assays were performed in duplicate for each value. Standard deviations are not depicted, as they were usually less than 1 %.

The inhibitory capacity of the multifunctional inhibitors towards MMP-2 was verified also by reverse zymography. As shown in Figure 15, all inhibitors prevented degradation of gelatin by MMP-2 in the gel matrix, leading to protein staining of non-degraded gelatin at the expected positions of the inhibitors. These bands are not visible in the control gel, which was loaded with the same amount of inhibitors (≈ 0.25 µg) but did not contain gelatin and MMP-2.
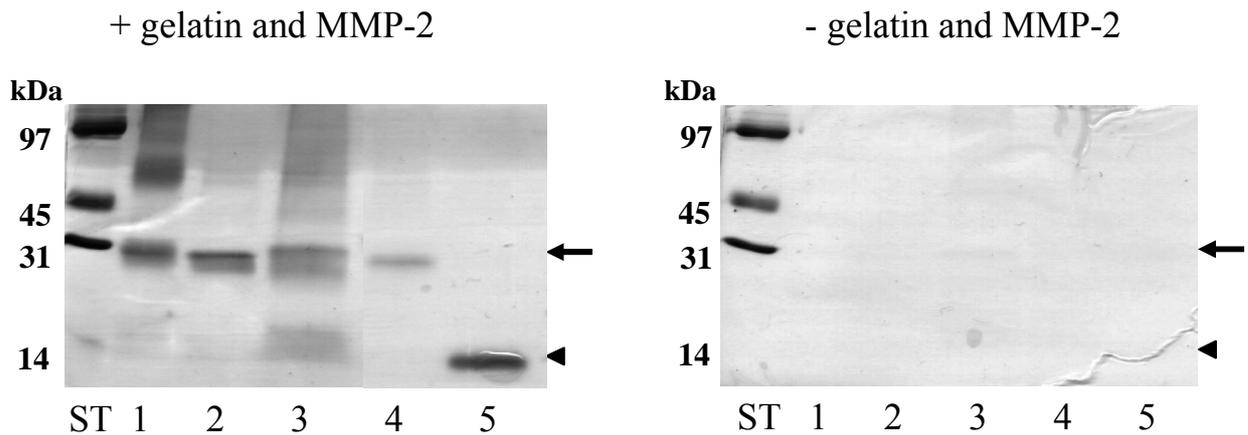

Figure 15. *Reverse zymography to analyse the inhibitory activity towards MMP-2.* **A.** About 0.25 µg of recombinant N-hTIMP-1-chCysWT (lane 1), N-hTIMP-1-chCys-uPA$_{19-31}$ (lane 2), N-hTIMP-3-chCysWT (lane 3), N-hTIMP-3-chCys-uPA$_{19-31}$ (lane 4), and N-hTIMP-1 (lane 5), respectively, were loaded into wells of an SDS polyacrylamide gel containing gelatin plus MMP-2. After electrophoresis, SDS was removed from the gel and the gel incubated overnight at 37 °C in 50 mM Tris–Cl, pH 7.5, 5 mM CaCl$_2$ and 5 µM ZnCl$_2$ for the digestion of the MMP-2 substrate, gelatin. Subsequently, the gel was stained with Coomassie blue. **B.** Control gel lacking gelatin and MMP-2.

4.4 Interaction of the Recombinant Inhibitors with Cell Surface-associated uPAR

To investigate whether N-hTIMP-1-chCys-uPA$_{19-31}$ (harboring the uPAR-binding region of uPA) interacts with cell surface-associated uPAR, PMA-stimulated U937 cells were incubated with fluorescently labeled high molecular weight uPA (FITC-HMW-uPA) mixed with various concentrations of purified, refolded N-hTIMP-1-chCys-uPA$_{19-31}$, and the resulting cell-associated fluorescence was determined. N-hTIMP-1-chCysWT (lacking the uPAR-binding site) served as the control protein. As shown in Figure 16, N-hTIMP-1-chCys-uPA$_{19-31}$ reduced binding of FITC-HMW-uPA to uPAR in a dose-dependent manner whereas N-hTIMP-1-chCysWT did not influence uPA/uPAR-interaction. The IC$_{50}$ -value of ATF (15 kDa) was calculated to be ~1.35 nM. In the case of N-hTIMP-1-chCys-uPA$_{19-31}$ (30 kDa), one has to consider that only 4% of the purified, refolded protein used in FACS analysis was active (as tested by cystatin activity). Taking this into account, the IC$_{50}$ -value (of the active protein) was ~ 6.7 nM, *i.e.* about 5-fold higher as that of the natural ligand ATF.


(ng per 250 µl per 250,000 cells)
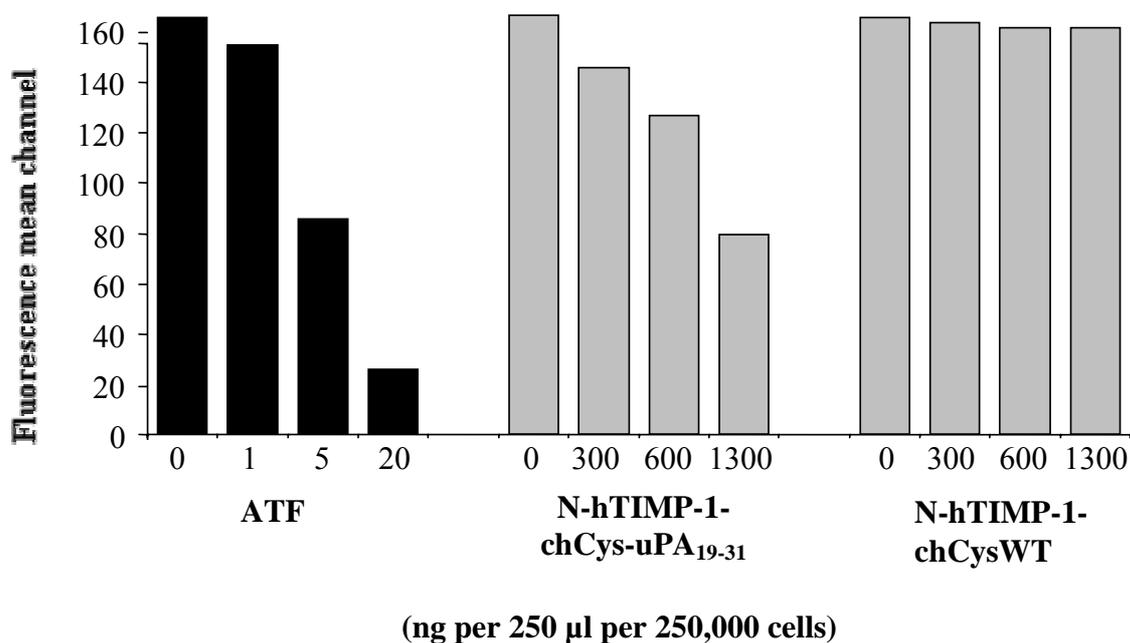
Figure 16. *Interaction of N-hTIMP-1-chCys-uPA$_{19-31}$ with cell surface-associated uPAR determined by Flow Cytofluorometry (FACS).* PMA-stimulated U937 cells were acid-washed to remove endogenous uPA. Then, cells were incubated with FITC-labeled HMW-uPA in the presence of refolded N-hTIMP-1-chCys-uPA19-31 or N-hTIMP-1-chCysWT and, subsequently, binding of labeled uPA to cell surface-associated uPAR was analyzed by flow cytofluorometry. N-hTIMP-1-chCysWT did not influence FITC-HMW-uPA binding, whereas N-hTIMP-1-chCys-uPA$_{19-31}$ significantly reduced uPA-binding to uPAR in a dose-dependent manner. The amino-terminal fragment of uPA (ATF) served as a positive control.The diagram depicts results of one representative experimen.

4.5 Simultaneous Interaction of the Recombinant Inhibitors with Papain and MMP-2

Interaction of the recombinant inhibitors with papain

The previous experiments showed that each part of the recombinant inhibitors retained its activity when fused together. The capacity of multifunctional inhibitors to simultaneously interact with the cysteine proteases and MMPs was assessed by surface plasmon resonance (SPR). For this, the cysteine protease papain was covalently bound to a SPR-sensor chip (CM-5). Then, N-hTIMP-1-chCysWT or N-hTIMP-1-chCys-uPA$_{19-31}$ were allowed to bind to the immobilized papain (Fig. 17A). As the binding constants for chCys/papain interaction lie in a fetomolar range there was almost no dissociation of recombinant inhibitors from papain even during intensive washing with buffer and the following binding of MMP-2 to these inhibitors could be analyzed.

Interaction of MMP-2 with the papain/inhibitor complex

After binding of multifunctional inhibitors to papain, activated MMP-2 was injected. As a control, chCysWT, that does not interact with MMPs, was bound to papain and also incubated with MMP-2. MMP-2 interacted only with the recombinant inhibitors harboring the N-terminal domain of TIMP-1, but not with chCysWT (Fig. 17B).

Different concentrations of activated MMP-2 were applied to the N-hTIMP-1-chCys-uPA$_{19-31}$/papain complex and a concentration-dependent binding was observed (Fig. 17C). Similar data were obtained with N-hTIMP-1-chCysWT (not shown). Applying the BIAcore evaluation software, the binding constants were calculated: the K_D of N-hTIMP-1-chCys-uPA$_{19-31}$ to MMP-2 (29.5 nM) was similar to that of N-hTIMP-1-chCysWT (32.0 nM). These values are in agreement with the K_D-value of activated MMP-2 to recombinant human TIMP-1 ($K_D = 28.6$ nM) previously reported by Olson *et al.* (1997).
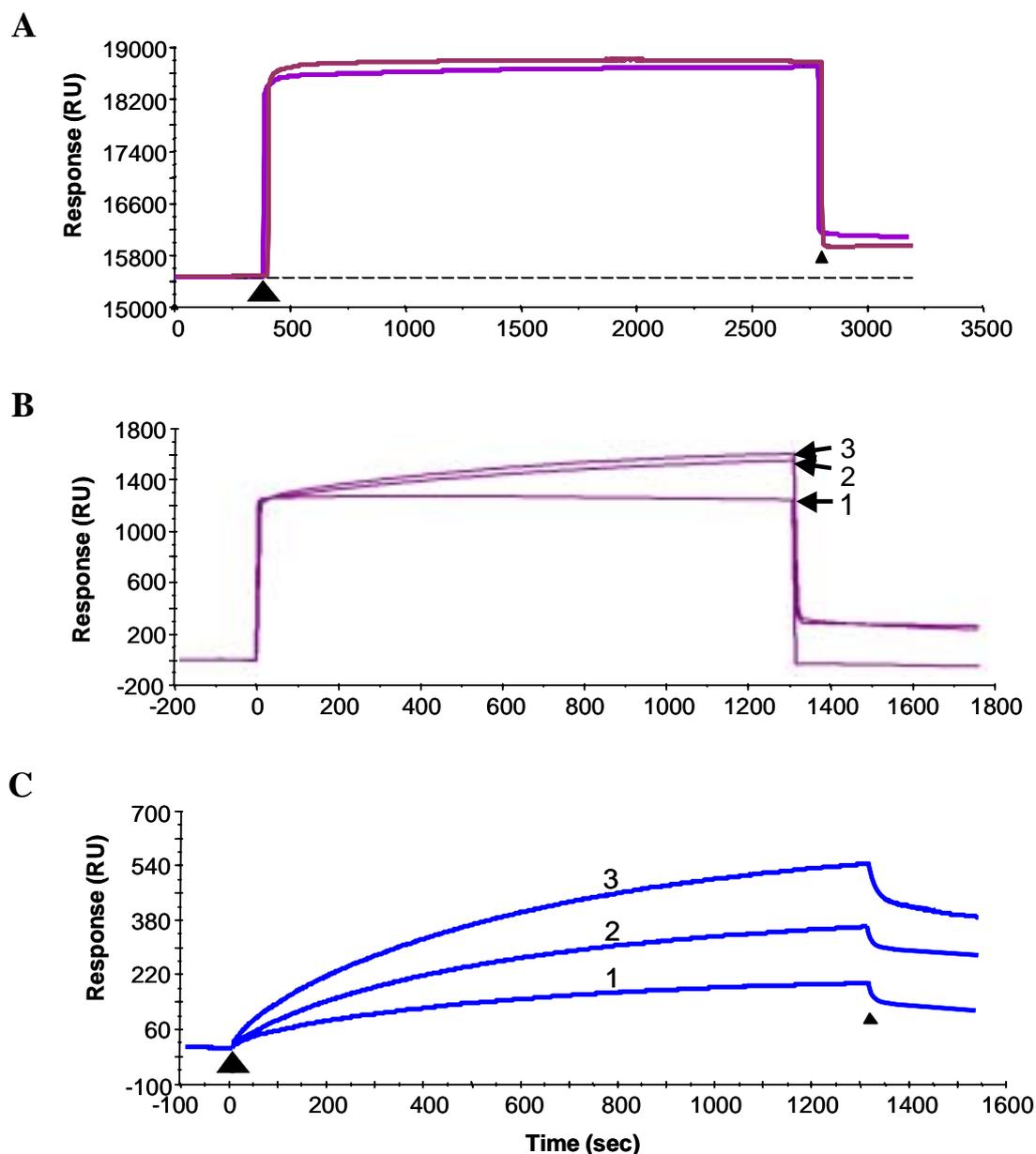
Figure 17. *Simultaneous Interaction of the Recombinant Inhibitors with Papain and MMP-2 Tested by Surface Plasmon Resonance Technology.* Approximately 2000 response units (RU) of papain were bound to a CM-5 chip inserted in the BIAcore 2000 system. Then, N-hTIMP-1-chCys-uPA$_{19-31}$, N-hTIMP-1-chCysWT, or chCysWT were injected for binding to papain. **A.** Binding of N-hTIMP-1-chCysWT and N-hTIMP-1-chCys-uPA$_{19-31}$ to papain (arrow: start of injection, arrowhead: end of injection). Both, the rise and drop off of the reference unit signal is mainly due to the change of buffer (bulk effect), whereas the difference in response (RU) before and after injection reflects the bound portion of chicken cystatin variant to immobilized papain. The very slow dissociation is due to the tight interaction with a K_i-value of about 2 pM (see Table 1) and a low dissociation rate constant, $k_{off} = 3.5 \times 10^{-5}$ (s^{-1}), determined with the fluorogenic substrate Z-Phe-Arg-AMC from presteady-state analysis (Machleidt *et al.*, 1993). **B.** Addition of activated MMP-2 to the papain-multifunctional inhibitor complexes results in binding to N-hTIMP-1-chCys-uPA$_{19-31}$ (curve 3) and to N-hTIMP-1-chCysWT (curve 2), but not to chCysWT (curve 1); the buffer bulk is not subtracted. **C.** Injection of different concentrations of activated MMP-2 (51, 68, and 102 μg/ml, curves 1-3, respectively) to the papain-N-hTIMP-1-chCys-uPA$_{19-31}$ complex; sensorgrams after subtraction of buffer bulk. Using these binding curves, the binding constant for MMP-2 binding to the recombinant inhibitor was calculated.

4.6 Generation of a Polyclonal Antibody against chCysWT in Rabbits

To produce an antibody against chCysWT two rabbits were immunized with purified, refolded recombinant chCysWT (Fig. 18A). Blood samples were taken on day 61, 100 and 130 from the first immunization and the sera were further tested by western blot analysis.

First, it was determined, whether the polyclonal antibody recognized chCys. Preliminary results showed that the anti-sera taken at all three time points reacted with purified, refolded chCysWT, chCys-uPA$_{19-31}$ and N-hTIMP-1-chCysWT (data not shown). As the imunogen used for the immunisation contained a (histidine)$_6$-tag, it was tested whether the polyclonal antibody showed crossreaction with other (histidine)$_6$-tag-containing proteins. For this, different (histidine)$_6$-tag-containing proteins were transferred onto the membrane and incubated with chCys anti-serum. The results presented in Figure 18B demonstrates that this polyclonal antibody selectively recognized chCysWT, chCys-uPA variants, and chCysWT or chCys-uPA variant fused with the N-terminal domain of TIMP-1/3, but not recombinant TF and uPAR (harboring a (histidine)$_6$-tag).
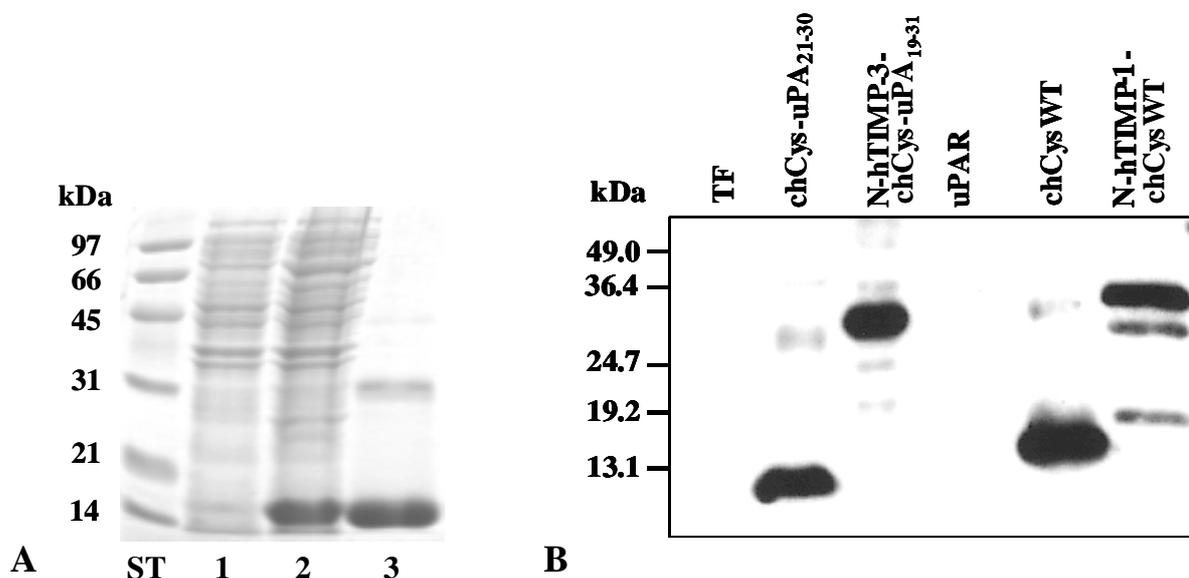

Figure 18. A. *Expression and purification of chCysWT.* Bacterial lysate befor (line 1) and after (line 2) induction of protein expression. The presence of the N-terminus (histidine)$_6$-tag allowed purification of the chCysWT by use of Ni^{2+}-NTA affinity chromatography, yielding a purity of > 95 % under denaturing and reducing conditions (line 3). **B. *Western blot analysis using anti-chCys polyclonal serum from day 61 after the first immunization.*** About 1µg of different (histidine)$_6$-tag fusion proteins were separated by 12 % polyacrylamide gel electrophoresis and transblotted. The membrane was incubated with anti-serum diluted 1:2000 and the reaction was followed by chemiluminescence. The polyclonal antibody recognized chCys-containing proteins and did not react with TF and uPAR, harboring a (histidine)$_6$-tag . Additional bands around 14 kD and 28 kD in the lane with N-hTIMP-1-chCysWT may correspond to the partially degraded protein.

4.7 Development of a New ELISA for Detection of the Multifunctional Inhibitors

For detection and quantification of the recombinant multifunctional inhibitors, a sandwich ELISA format was developed in a similar fashion as described previously (Kotzsch *et al.,* 2000). After optimization the finally used protocol was as follows: 96-well plates were coated with rabbit polyclonal anti-chCysWT antibodies (day 61), diluted 1:2000 in 50 mM carbonate buffer pH 9.6, and incubated at 4 °C overnight. The next day, plates were washed 3 times with wash buffer (50 mM potassium phosphate, pH 7.2; 150 mM NaCl; 0.05 % Tween 20) and blocked with Microplate Blocking Reagent (Qiagen, Hilden, Germany) for 2 h at RT. After one washing step, the standards, control, or samples (all diluted in PBS/0.2% BSA) were added to the plates and incubated for 2 h at RT. Then, the plates were washed 3 times with wash buffer and 100 µl Penta-His-HRP Conjugate (Qiagen, Hilden, Germany), diluted 1:2000 in PBS/0.2% BSA, were given to each well. After 2 h incubation at RT the plates were again washed with wash buffer and 100µl of TMB Peroxidase Substrate (KPL, Gaithersburg, Maryland, USA) was added to each well. The enzymatic reaction was stopped after 30 min with 0.5 N H_2SO_4 and the absorbance was read with a microtest plate reader at a wavelength of 450 nm. A representative standard curve of this ELISA is shown in Figure 19. The lower detection limit was 0.25 ng/ml.
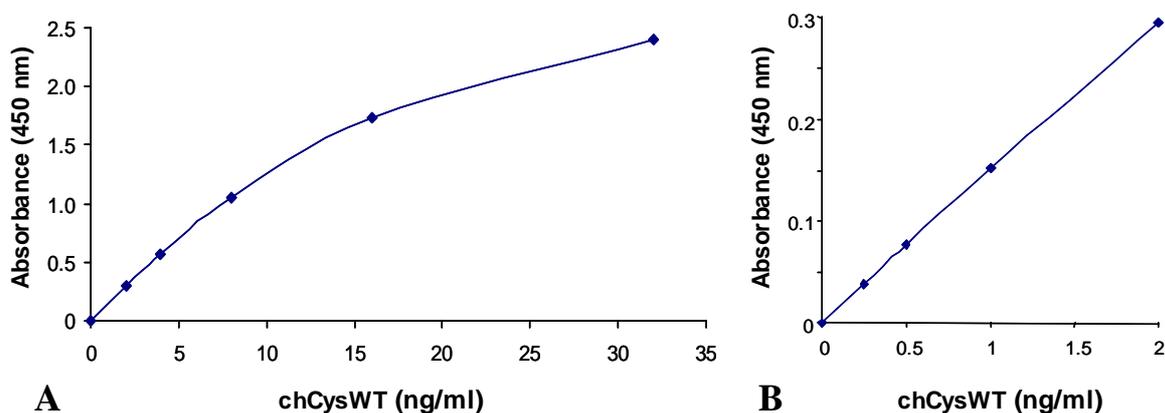

Figure 19. *A typical standard curve of the chCys-ELISA for detection of the multifunctional inhibitors using recombinant chCysWT as the standard.***(A)** Serial dilutions of purified recombinant chCysWT covering a concentration range of 2 to 32 ng/ml **(B)** Serial dilutions of purified recombinant chCysWT covering a concentration range of 0.25 to 2 ng/ml.

The reproducibility of the new chCys-ELISA was estimated using cell culture supernatantes from the transfected ovarian cancer cells, expressing multifunctional inhibitors. The coefficients of variations (CVs) of the inter-assay precision were 7.5-14 (Table 2).

	n	Mean (ng/ml)	SD (ng/ml)	CV (%)
N-hTIMP-1-chCysWT	3	4.65	0.35	7.5
N-hTIMP-1-chCys-uPA$_{19-31}$	3	1.05	0.15	14.0
N-hTIMP-3-chCysWT	3	1.70	0.20	11.7
N-hTIMP-3-chCys-uPA$_{19-31}$	3	8.95	0.95	10.6

Table 2. *Precision of the chCys-ELISA.* Inter-assay precision analysis was performed using cell culture supernatantes from the transfected ovarian cancer cells, expressing multifunctional inhibitors.

4.8 Expression of Multifunctional Inhibitors

Ovarian cancer OV-MZ-6#8 cell lines were generated which express and secrete the novel bi- and trifunctional inhibitors. For this, the cells were stably transfected with pRc-RSV-derived expression plasmids encoding either pre-N-hTIMP-1-chCysWT, pre-N-hTIMP-1-chCys-uPA$_{19-31}$, pre-N-hTIMP-3-chCysWT, or pre-N-hTIMP-3-chCys-uPA$_{19-31}$. As a control OV-MZ-6#8 cells were transfected with the empty vector only. To prove synthesis and secretion of the recombinant proteins, cell culture supernatant, diluted 1:2 in PBS/0.2% BSA, was subjected to a new ELISA, which employs the polyclonal antibody to recombinant chCysWT as the catching antibody and a peroxidase-coupled monoclonal antibody directed against a (histidine)$_5$-epitope as the detection antibody. All stably transfected cell lines produced and secreted detectable amounts of the respective multifunctional inhibitor into the medium, with amounts ranging from 3 to 21 ng/10^5 cells secreted into the medium after growth for 48 h (Fig. 20). No reaction above background (medium only) was observed by testing the conditioned medium of vector-transfected cells (Fig.20).
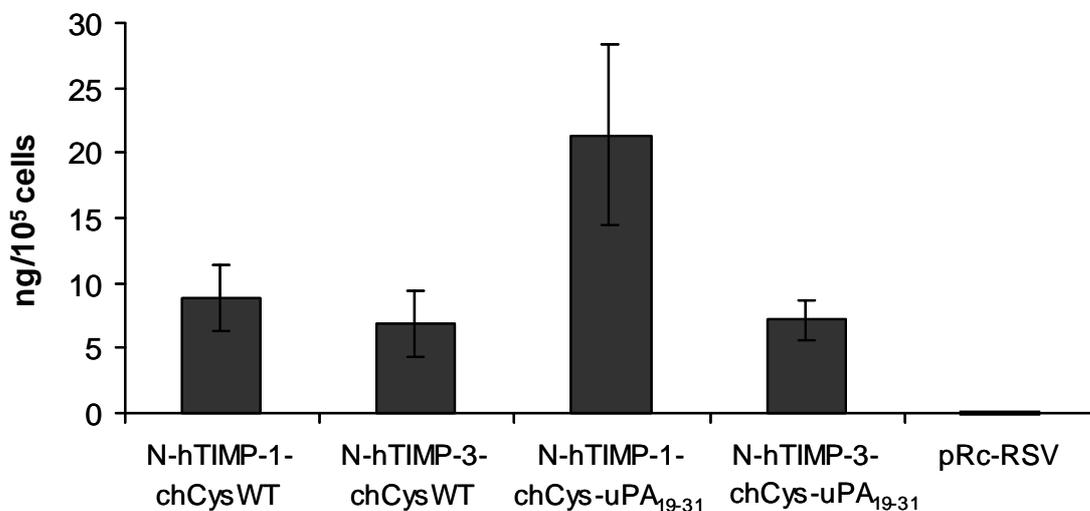
Figure 20. *Secretion of multifunctional inhibitors by transfected cells into cell culture medium, determined by ELISA.* 30,000 cells/well were subcultured in a 24-well plate for 48 h. Then, cell culture supernatants were added to the microtiter plate precoated with rabbit anti-chCys polyclonal antibodies. As a detection andibody a peroxidase-coupled monoclonal antibody directed against a (histidine)$_5$-epitope were used. All transfected cell lines produced and secreted detectable amounts of the respective multifunctional inhibitor into the medium, with amounts ranging from 6 to 29 ng/10^5 cells after growth for 48 h. No reaction was observed by testing the conditioned medium of vector-transfected cells. The assays were performed in duplicate for each value. Standard deviations are not depicted, as they were usually less than 1 %.

4.9 Characterization of Proliferation of the Cells Expressing Multifunctional Inhibitors

Proliferation of the stably transfected cells was tested under the normal culturing conditions. Cells were subcultured in serum-containing medium in 24-well plate, for 48 and 96 h and then counted using a Neubauer hemocytometer. As shown in Figure 21, expression of the recombinant inhibitors had no influence on cell proliferation.
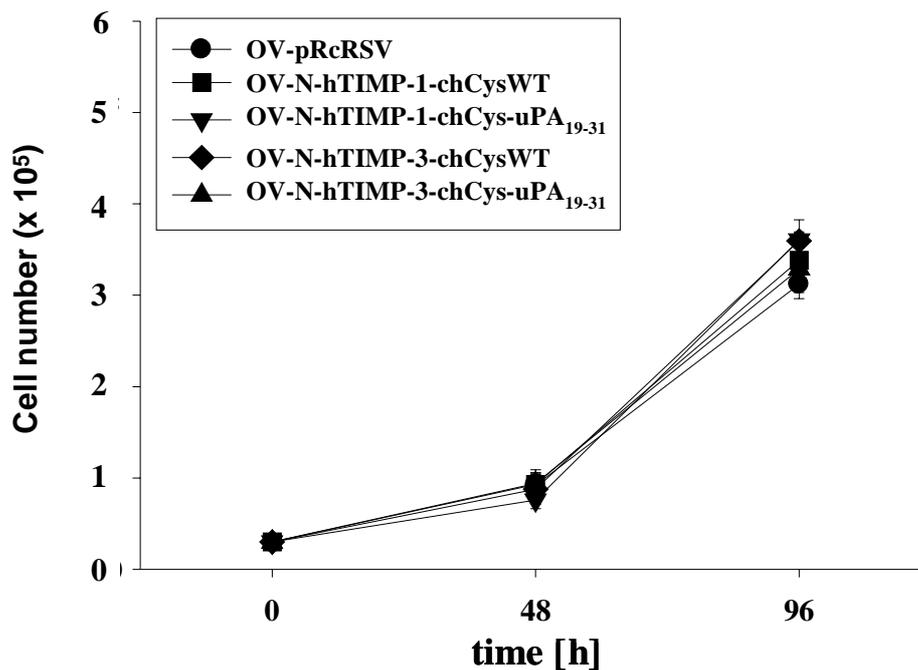
Figure 21. *Proliferation of OV-MZ-6#8-derived cells, either expresing multifunctional inhibitors N-hTIMP-1-chCysWT, N-hTIMP-3-chCysWT, N-hTIMP-1-chCys-uPA$_{19-31}$ and, N-hTIMP-3-chCys-uPA$_{19-31}$ or transfected with vector only.* Proliferation of each of these cell lines was tested by seeding of 30,000 cells in 1 ml DMEM, 10% (v/v) FCS into wells of a 24-well plate. After 48 or 96 h of incubation, cells were counted in Neubauer hemocytometer upon Trypan blue exclusion. No difference in growth between transfected cells and vector control was detected. Four independent experiments were performed in triplicate. Mean values ± SD are indicated.

4.10 Determination of the Adhesive Capacities of the Transfected Cells to Different ECM-Proteins

In oder to examine the effect of transfection on cell adhesion, cells stably transfected with expression plasmids encoding the multifunctional inhibitors or vector were seeded into wells of microtiter plates precoated with fibronectin, vitronectin, or collagen type IV. Adherent cells were then detected using a hexoaminidase-substrate. Cell lines expressing N-hTIMP-1-chCysWT, N-hTIMP-3-chCysWT, and N-hTIMP-3-chCys-uPA$_{19-31}$ showed no difference in adhesion to all tested ECM proteins in comparison to vector control. In the case of cells expressing N-hTIMP-1-chCys-uPA$_{19-31}$ a slightly increased adhesion to fibronectin, vitronectin and collagen type IV was observed (Fig. 22).
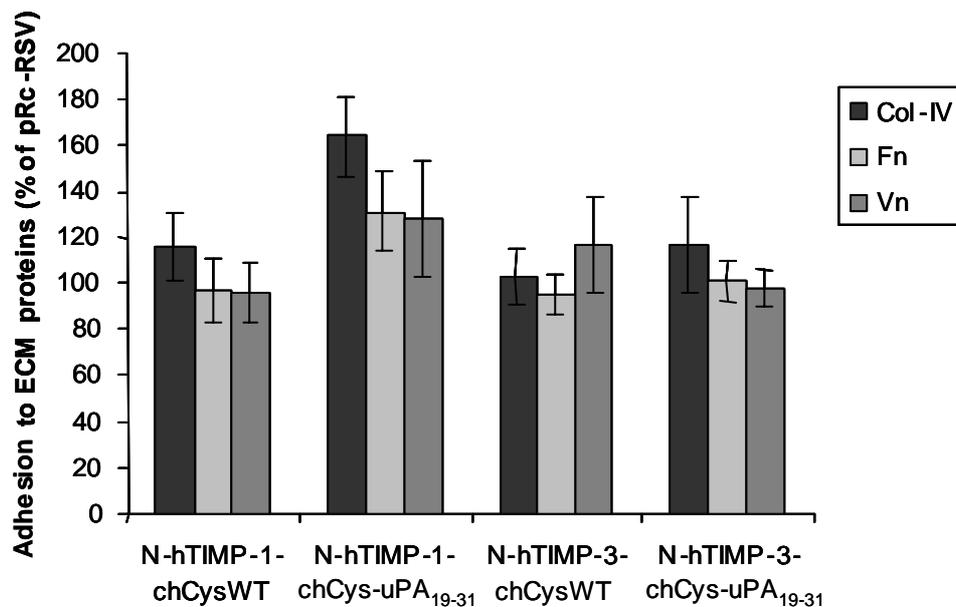
Figure 22. *Adhesion of cells expressing multifunctional inhibitors to different ECM proteins.* Cells stably transfected with the expression plasmids encoding multofunctional inhibitors or vector were seeded into the wells of an 96-well plate precoated with vitronectin, fibronectin or collagen type IV. After 2 h of incubation, non-adherent cells were washed out with PSB and the remaining cells were incubated with a hexoaminidase substrate solution. The resulting color reaction was measured on a microtest plate reader at a wavelength of 405 nm. Values are given in relation to the adhesive capacity of pRc-RSV-vector cells, which was set to 100 %. Expression of the multifunctional inhibitors N-hTIMP-1-chCysWT, N-hTIMP-3-chCysWT, and N-hTIMP-3-chCys-uPA$_{19-31}$ had no influence on cell adhesion to the tested ECM proteins, whereas expression of N-hTIMP-1-chCys-uPA$_{19-31}$ resulted in a slight enhancement of cell adhesion. Mean values ± SD of four independent experiments are indicated (each experiment was performed in triplicate).

4.11 Biological Activity of the Multifunctional Inhibitors in *in vitro* Invasion Assays

To analyze the effects of the recombinantly expressed multifunctional inhibitors on the invasive capacity of human ovarian cancer cells, we performed *in vitro* Matrigel invasion assays. Stably transfected cells were placed into the upper compartment of an invasion chamber on top of a Matrigel-coated filter, the lower compartment was filled with fetal calf serum (FCS)-containing medium as a chemoattractant. After 48 h, the cells on the lower side of the filter were counted. The results, presented in Figure 23A, demonstrate that the inhibitor-producing cells exhibit a significant decrease in invasion through Matrigel in comparison to the vector-transfected control cells. A direct quantitative comparison of the effectiveness of the different inhibitors expressed by the ovarian cancer cells is not possible, since (a) the cell clones produce varying amounts of the recombinant inhibitors and (b) the portion of active recombinant protein is not known.

In another set of experiments, purified and refolded recombinant N-hTIMP-1-chCysWT and N-hTIMP-1-chCys-uPA$_{19\text{-}31}$, produced in *E. coli*, were added exogenously to wild-type OV-MZ-6#8 cells. Again, the inhibitors inhibited invasion of the cancer cells through the Matrigel-coated filter (Fig. 23B). In these experiments, N-hTIMP-1-chCys-uPA$_{19\text{-}31}$ reduced tumor cell migration to a lower extent than the bifunctional hybrid N-hTIMP-1-chCysWT, very likely due to the fact that the active concentration of the chCysWT-harboring fusion protein was much higher as compared to the chCys-uPA$_{19\text{-}31}$ variant ($\approx 8\%$ versus $\approx 4\%$).

A

B
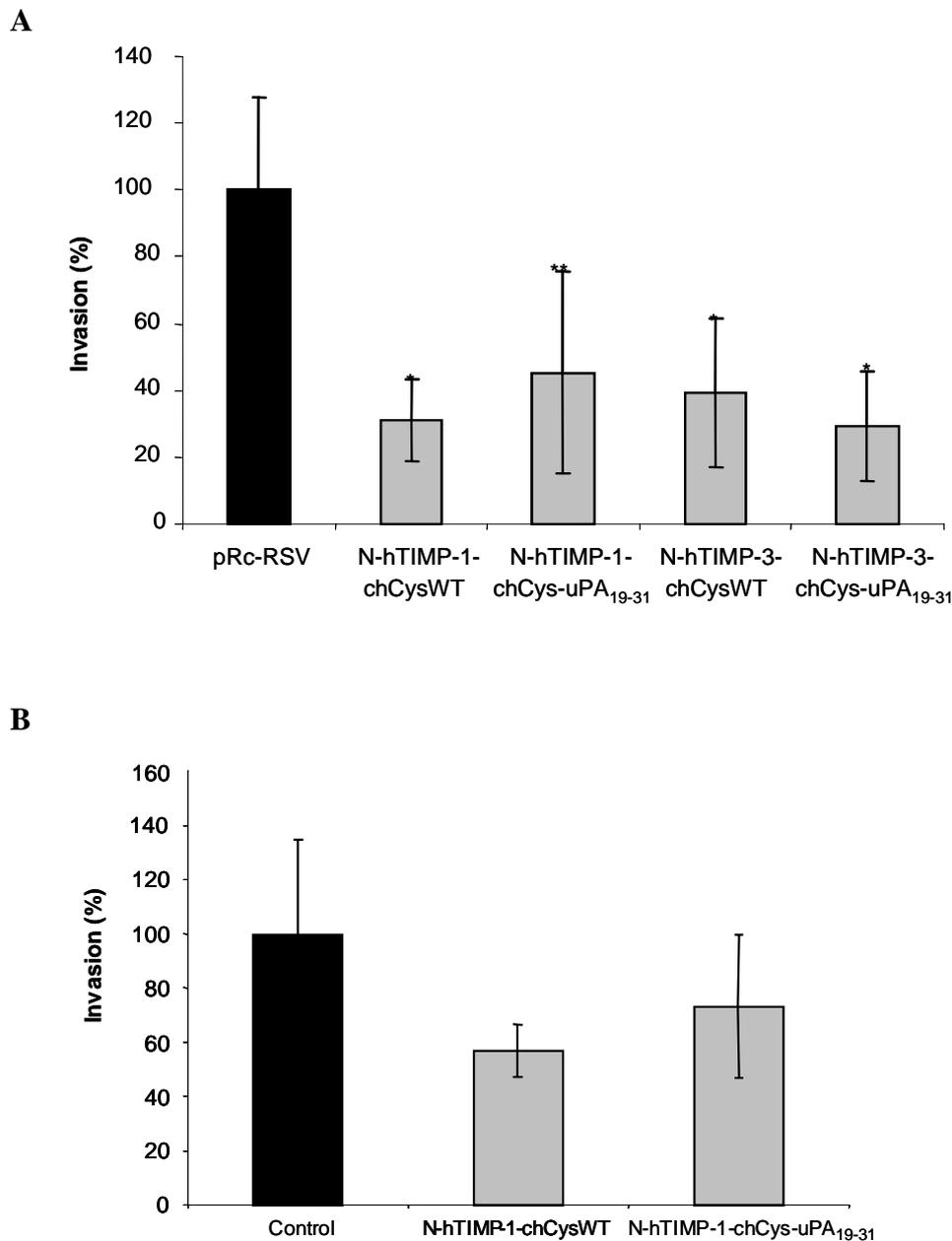
Figure 23. *In vitro* **Matrigel invasion assays. A.** Stably transfected ovarian cancer OV-MZ-6# 8 cells were placed into the upper compartments of the invasion chambers (5×10^4 cells in 500 µl 0.1 % BSA/DMEM pro insert). The lower chambers of the inserts were filled with 750 µl DMEM containing 10 % FCS as the chemoattractant. After 48 h incubation, the Matrigel layer *plus* noninvaded cells of the upper compartment was wiped off, the invaded cells on the lower side of the filter were fixed, stained, and counted. Assays were performed in triplicate. The presented diagram shows results of three independent experiments. *, p<0.001; **, p<0.05. The data were analyzed for statistical significance by a Student's *t*-test. **B.** Invasion assays with recombinant purified and refolded inhibitors isolated from *E. coli*. Wild type OV-MZ-6# 8 cells (5×10^4 cells in 400 µl) were applied to each upper compartment and recombinant inhibitors ($\approx$ 50 µg protein in 150 µl DMEM) were added to the cells together with 500 µg BSA. The controls received PBS with 500 µg BSA. The diagram depicts results of one representative experiment performed in triplicate.

4.12 Effect of Multifunctional Inhibitors on Tumor Growth and Spread *in vivo*

The effect of endogenously expressed multifunctional inhibitors on primary tumor growth and spread was analyzed in a *xenograft nude* mouse model. Each mouse received 7 x 10^6 cells of either OV-N-hTIMP-1-chCysWT, OV-N-hTIMP-1-chCys-uPA$_{19-31}$, OV-N-hTIMP-3-chCysWT, or OV-N-hTIMP-3-chCys-uPA$_{19-31}$, which were inoculated intraperitoneally. As a control, one group of mice was inoculated with OV-pRcRSV cells. After 56 days, tumor mass within the peritoneal cavity, appearing as focal tumors located below the liver, in the mesenterium, and tumor cell layers or colonies along the diaphragm and the inner abdominal wall (*peritoneum parientalis*), was determined. Significant reduction of tumor mass was observed in mice inoculated with the cell lines expressing bifunctional inhibitors (OV-N-hTIMP-1-chCysWT; 72 % reduction; OV-N-hTIMP-3-chCysWT; 75 % reduction) as well as the cell line expressing the trifunctional inhibitor N-hTIMP-1-chCys-uPA$_{19-31}$ (71 % reduction), in comparison to the tumor mass generated by OV-pRcRSV cells (Fig. 24). Expression of N-hTIMP-3-chCys-uPA$_{19-31}$ also led to the formation of a smaller intraperitoneal tumor mass (54 % reduction) as compared to the control, however, in the latter case, the differences were not significant. By comparison of the four groups expressing the different therapeutic molecules, no significant anti-tumorigenic advantage could be assigned to the expression of one particular multifunctional inhibitor.

In addition to the evaluation of the relative tumor mass in the intraperitoneal cavity, differences of the localization of tumor cells along the areas of the diaphragm and the inner abdominal wall were analyzed. A distinguishable pattern was observed in mice inoculated with cells secreting multifunctional inhibitors as compared to OV-pRcRSV control cells. In the case of OV-pRcRSV cells, numerous clearly visible tumor cell colonies spreading over the inner abdominal wall and extended tumor layers on the diaphragm were found (Fig. 25). In the case of the OV-MZ-6#8 cells expressing the multifunctional inhibitors, a reduction in the number and size of tumor cell colonies spreading over the inner abdominal wall was observed. In mice inoculated with cell lines expressing N-hTIMP-1-chCysWT, N-hTIMP-1-chCys-uPA$_{19-31}$, and N-hTIMP-3-chCysWT, the analysis additionally revealed a distinct reduction of tumor areas on the diaphragm (Fig. 25).
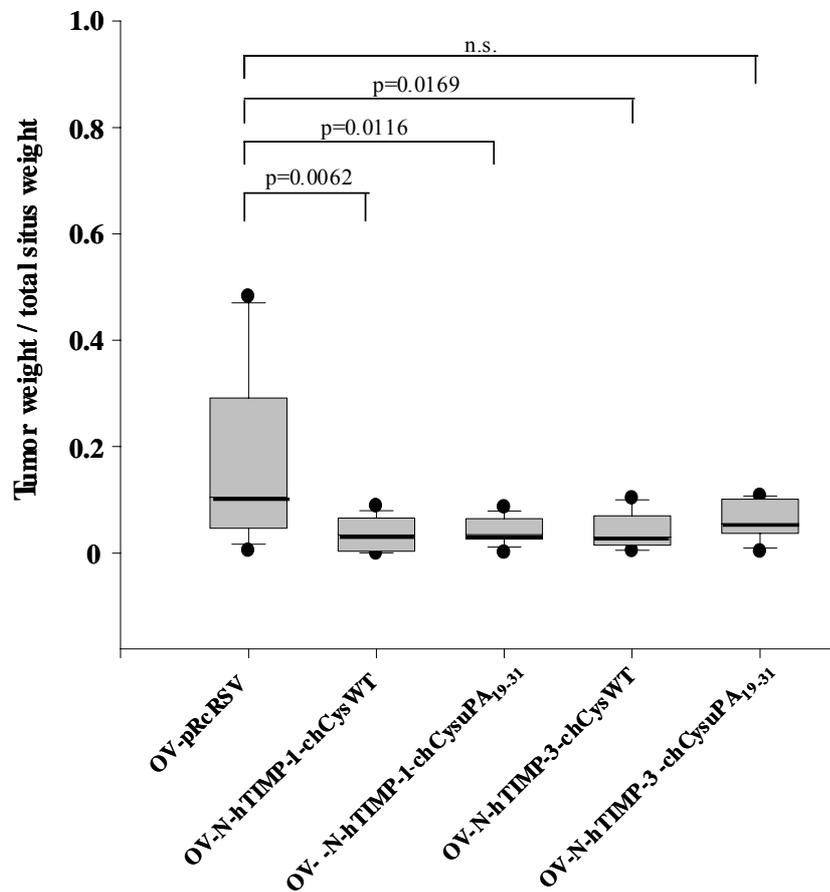
Figure 24. *In vivo* **t***umor growth of ovarian cancer cells synthesizing multifunctional inhibitors.* 7 x 10^6 OV-N-hTIMP-1-chCysWT, OV-N-hTIMP-1-chCys-uPA$_{19-31}$, OV-N-hTIMP-3-chCysWT, OV-N-hTIMP-3-chCys-uPA$_{19-31}$, and OV-pRcRSV cells were inoculated into the peritoneal cavity of female athymic (*nu/nu*, CD41) mice. After 56 days, the mice were sacrificed and the relative tumor mass within the total situs determined. Four different OV-MZ-6#8-derived cell lines, expressing either trifunctional inhibitors, OV-N-hTIMP-1-chCys-uPA$_{19-31}$ (n=9; median tumor proportion (m.t.p.)=0.0347) and OV-N-hTIMP-3-chCys-uPA$_{19-31}$ (n=7; m.t.p.=0.0556), or bifunctional inhibitors, OV-N-hTIMP-1-chCysWT (n=9; m.t.p.=0.0343) and OV-N-hTIMP-3-chCysWT (n=6; m.t.p.=0.0299) were compared to control cell line OV-pRcRSV (n=13; m.t.p.=0.1217), stably transfected with the expression plasmid only. The box plot marks the 25th and 75th percentile, the vertical bars above and below indicate the 10th and the 90th percentile, respectively. The median value is indicated by a bold bar. The dots present the statistical significance. p<0.05 was considered statistically significant; n.s., not significant.
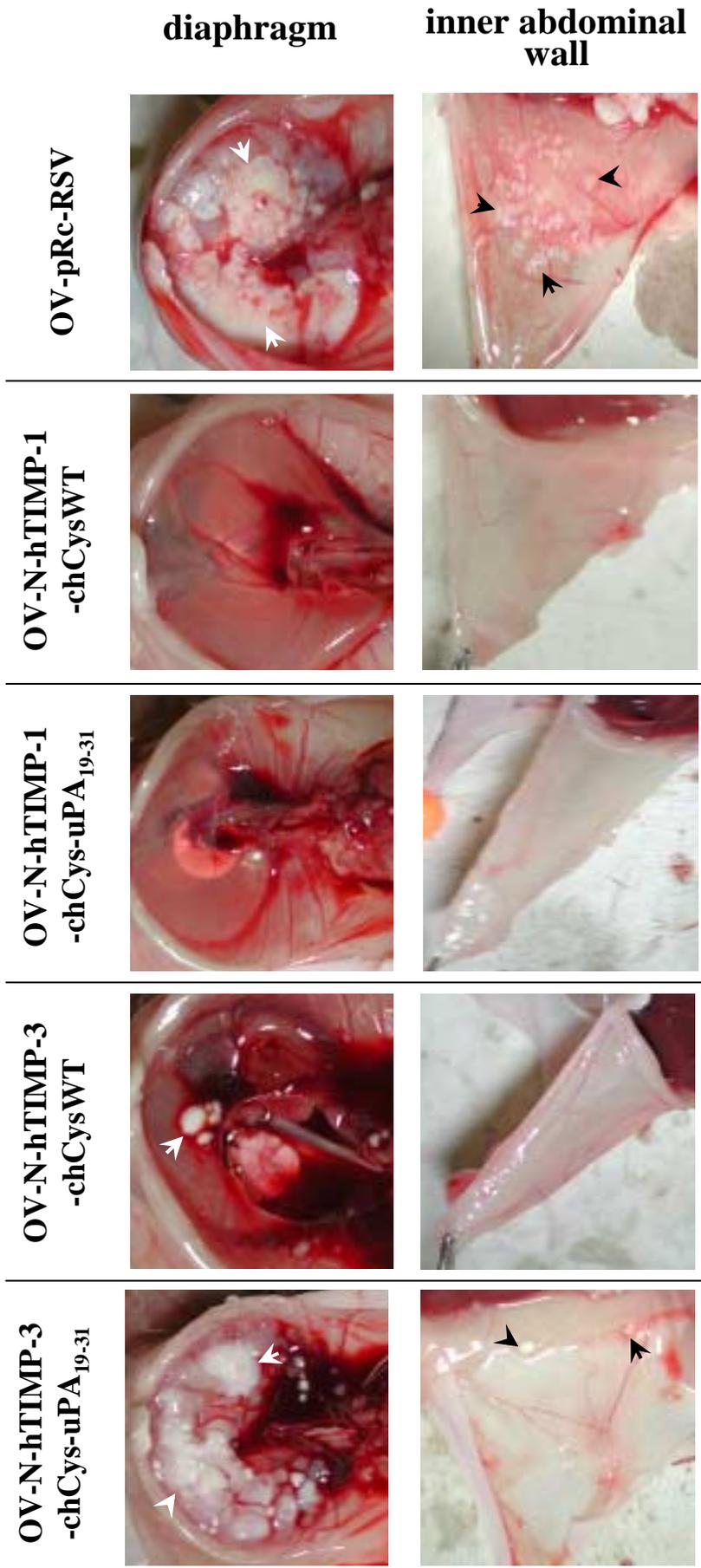
Figure 25. *Tumor spread at the diaphragm and inner abdominal wall.* Tumors spread of representative mice from groups inoculated with OV-pRcRSV cells and OV-cell lines expressing multifunctional inhibitors N-hTIMP-1-chCysWT, N-hTIMP-1-chCys-uPA$_{19\text{-}31}$, N-hTIMP-3-chCysWT, and N-hTIMP-3-chCys-uPA$_{19\text{-}31}$. The extent of tumor cell colonies (arrow heads) spreading across the diaphragm was drastically reduced in the case of OV-N-hTIMP-1-chCysWT, OV-N-hTIMP-1-chCys-uPA$_{19\text{-}31}$, and OV-N-hTIMP-3-chCysWT. Spreading across the inner abdominal wall was distinctly reduced in all mice injected with multifunctional inhibitor-expressing cells.

4.13 Detection of the Inhibitor-Antigen Level in Tumor-Tissue Extracts, Blood and Ascitic Fluid of Nude Mice, Intraperitoneally Inoculated with OV-MZ-6#8-Derived Cell Lines Synthesizing Multifunctional Inhibitors

To prove, whether the transfected tumor cells synthesized the multifunctional inhibitors *in vivo*, tumor tissue, blood, and ascites were obtained from sacrificed mice. The content of multifunctional inhibitors was determined by ELISA, which selectively detects the recombinant proteins. Inhibitor-antigen was detectable in all tested tumor tissues and ascitic fluids (summarized in Table 3), whereas no inhibitors were found in blood samples. The highest amount of inhibitor-antigen was detected in tumor tissue originating from OV-N-hTIMP-1-chCys-uPA$_{19-31}$ cells. These data reflect the feature of this cell line *in vitro*, where the highest secretion of inhibitor into cell culture supernatant was achieved by OV-N-hTIMP-1-chCys-uPA$_{19-31}$ cells. Antigen levels obtained in tumor tissues and ascitic fluids of the remaining three expressing multifunctional inhibitor groups are almost identical. The detected high amounts of these inhibitors in the ascitic fluids of tumor bearing mice indicate a remarkable stability of these hybrid proteins *in vivo* (Tab. 3).

	Tumor-tissue extract (ng/mg total protein)		Ascitic fluid (ng/ml)	
pRc-RSV	0.03 ± 0.05	(n=3)	1.02 ± 1.0	(n=6)
N-hTIMP-1-chCysWT	1.30 ± 0.4	(n=3)	94.30 ± 81.0	(n=3)
N-hTIMP-1-chCys-uPA$_{19-31}$	32.80 ± 28.9	(n=3)	143.50 ± 100	(n=3)
N-hTIMP-3-chCysWT	0.60 ± 0.3	(n=3)	13.90 ± 14.5	(n=3)
N-hTIMP-3-chCys-uPA$_{19-31}$	1.40 ± 0.4	(n=3)	20.50 ± 11.4	(n=5)

Table 3. *Antigen levels of multifunctional inhibitors present in tumor extracts of ovarian tumor tissue, and in ascitic fluid.* Synthesis and secretion of the inhibitors in cell line supernatants, tumor extracts, and ascitic fluids was proven by an ELISA, which selectively detects the recombinant proteins. Mouse tumor tissue extracts were prepared by suspending pulverized tissue previously frozen in liquid nitrogen, in TBS, 1 % (*v/v*) Triton X-100, followed by centrifugation (60 min, 13,000 x *g*, 4 °C). Supernatants were subjected to ELISA. Total protein was determined by BCA^{TM} Quantitation Kit. Ascitic fluids were collected from the peritoneal cavities of tumor bearing mice 56 days after tumor cell inoculation.

5. Discussion

An important step in tumor invasion and metastasis is the degradation of extracellular matrix, allowing cells to migrate through the connective tissue and the basement membranes. These processes involve the action and interaction of several different proteolytic systems such as cysteine proteases, serine proteases, and MMPs. *In vivo*, all of these protease systems are controlled by specific natural inhibitors, *e.g.* MMPs by the TIMP-family and extracellular cysteine proteases by cystatin C (for reviews see: Stamenkovic, 2000; Andreasen *et al.,* 2000; Kos and Lah, 1998; Johansson *et al.,* 2000; Ellis and Murphy, 2001; Magdolen *et al.,* 2002). Due to the essential role of proteolytic systems in tumor invasion and metastasis, synthetic inhibitors were developed to be used as therapeutic agents to affect tumor progression (Coussens *et al.,* 2002; Sperl *et al.,* 2001). Still, all of these natural and synthetic inhibitors are directed against only one of the mentioned protease systems. Thus, a promising new approach in tumor therapy is to apply multifunctional inhibitors, which should reduce the activity of several or all of these protease systems involved in tumor progression.

Based on this idea, a novel bifunctional inhibitor chCys-uPA$_{19-31}$ was developed, which reduces the activity of cysteine proteases, such as cathepsin B and L, and simultaneously also prevents binding of uPA to its receptor uPAR (Muehlenweg *et al.,* 2000). In the present study this strategy was extended and multifunctional inhibitors, also directed against MMPs, were designed. To construct these inhibitors, the N-terminal domains of TIMP-1 and TIMP-3 were used. It is known that only the N-terminal domain of TIMPs is necessary for MMP inhibition and that this domain retains metalloproteinase inhibitory activity even when expressed as a single entity (Murphy *et al.* 1991; Nguyen *et al.*, 1994). Additionally, the C-terminal domain of TIMP-1 is implicated in the formation of a specific non-inhibitory complex with the zymogen of MMP-9 (pro-MMP-9) that is thought to play a role in pro-MMP-9 activation (Goldberg *et al.*, 1992). Structural studies on MMP-3/TIMP-1 interaction performed by Bode and his colleagues (2000) demonstrated that the N-terminal aa Cys-1 to Val-4 are essential for protease-inhibitor interaction. A key feature of this process is the bidentate coordination of the catalytic site Zn^{2+} of MMP-3 by the α-amino and carbonyl groups of the N-terminal Cys-1 of TIMP-1 while the side chain of Thr-2 projects into the S' pocket of the metalloproteinase. Refinement of the structure indicates that the –OH group of this residue interacts with Glu-202 of MMP-3 and displaces a water molecule from the active site that normally play a key role in catalysis (Gomis-Rüth *et al.*, 1997; Huang *et al.*, 1997). Therefore, taking into consideration the importance of the first N-terminal residues of TIMPs in MMP inhibition, the

chCysWT or the chCys-uPA variant was recombinantly added to the C-terminus of N-TIMP. The two inhibitor domains were connected through a flexible linker that makes them relatively independent from each other.

The recombinant bi- and trifunctional inhibitors were expressed in *E. coli,* purified, and refolded into an active conformation. However, the highest yield of the active inhibitor that could be achieved was about 8% of total protein for N-hTIMP-1-chCysWT and 4% for N-hTIMP-1-chCys-uPA$_{19-31}$. The reason for this inefficient refolding is most likely due to the formation of incorrect disulfide linkages between cysteine residues in a semi-folded nascent polypeptide (Lee *et al.*, 2001). The recombinant multifunctional inhibitors contain six disulfide bridges (three in the N-terminal domain of TIMP-1/-3 and three in chCys), that have to be correctly formed during the refolding process (Magdolen *et al.*, 2002). Despite attempts to improve the refolding efficiency, the yield of active N-hTIMP-3-containing inhibitors was significantly lower than those for fusion proteins harboring the N-hTIMP-1 domain. Difficulties in purification of TIMP-3 and N-TIMP-3 were reported by several researches and this is also a reason for the absence of structural investigations on TIMP-3. Analysis of the refolding profiles of N-TIMP-1 and N-TIMP-3 (Lee *et al.*, 2002) revealed that the refolding pathway of N-TIMP-3 is different from that of N-TIMP-1, and N-TIMP-3 refolding may be dominated by energetically unstable intermediates, which render the efficiency of refolding unfavorable. In preparations of our fusion proteins harboring the chCys-uPA$_{19-31}$-moiety, generally, a smaller amount of active protein was present compared to chCysWT-containing molecules, most probably due to a less effective refolding at the uPA$_{19-31}$-containing proteins.

Quantitative evaluation of the inhibitory capacities of the recombinant inhibitors demonstrated that the individual inhibitory domains are similarly potent as their natural counterparts: i) The K_i-values (1.4 pM - 2 pM) of the inhibitors against the cysteine protease papain are very close to the inhibition constant (1.4 pM) of natural wild-type cystatin (Machleidt *et al.,* 1993). ii) The binding affinity of the TIMP-variants to MMP-2 as tested by SPR (K_D of about 30 nM) is also similar to that of TIMP-1 with MMP-2 (Olson *et al.*, 1997). iii) The binding affinity of uPA$_{19-31}$-containing cystatin variants towards uPAR is in the range of uPA to uPAR when analyzed by SPR (5 nM *versus* 17 nM; Muehlenweg *et al.,* 2000). These results strongly indicate that in the bi- and trifunctional inhibitors the functional integrity of each individual module is not significantly affected when recombinantly fused together. Thus, these multifunctional inhibitors may represent suitable tools to efficiently inhibit members of either of the three targeted protease families.

Previously, it was demonstrated that the bifunctional inhibitor chCys-uPA$_{19-31}$ inhibits cysteine proteases and at the same time interferes with uPA/uPAR-interaction (Muehlenweg *et al.,* 2000). The same is true for the novel bi- and trifunctional inhibitors with respect to their interaction with cysteine proteases and MMPs as shown by SPR technology. Although the multifunctional inhibitors have the capacity to inhibit simultaneously more than one of their target molecules, it is not imperative that a single trifunctional inhibitor can inhibit all three of the target molecules at the same time. Rather, it is only important that each molecule carries the potential to inhibit one of the three systems. The characterization of recombinant purified inhibitors, although clearly demonstrating their inhibitory activity, was hampered by the fact that a major part of the purified proteins was inactive, mainly owing to misfolding by the refolding procedure after expression in *E. coli*. Therefore, for further investigation of the biological effects of multifunctional inhibitors, ovarian cancer cells OV-MZ-6#8 were stably transfected with the expression plasmids encoding the various recombinant inhibitors. The expression levels that are achieved using mammalian cells is usually low in comparison with the *E. coli* expression systems. Therefore, sensitive detection methods, such as specific antibodies are needed to verify the expression and secretion of the recombinant proteins into cell culture supernatant by immunological methods. Commercially available polyclonal antibodies directed against TIMPs recognize the C-terminal domain of TIMPs and do not react with the N-terminal domain. Polyclonal or monoclonal antibodies directed against chCys were also not available. To produce polyclonal antibodies, two rabbits were immunized with recombinant chCysWT, harboring a (histidine)$_6$-tag. Western blot analysis showed that this polyclonal antibody is specific for chCys and does not cross-react with other (histidine)$_6$-tag-containing control proteins. Additionally, these antibodies were used as the catching antibody for the development of a sandwich ELISA system, with a peroxidase-coupled monoclonal antibody directed against a (histidine)$_5$-epitope as the detection antibody. This ELISA system showed sufficient precision and sensitivity (0,25 ng/ml), and selectively detects the recombinant inhibitors.

To test whether the expression of the multifunctional inhibitors influenced biological characteristics of the transfected cell, *in vitro* proliferation, adhesion and invasion assays were performed. In recent years, a mitogen-like function has been described for uPA, which appears to be highly cell-type-specific regarding the requirement of structural elements within the uPA molecule. Thus, Rabbani *et al*. (1992) showed a mitogenic activity of both enzymatically active and inactive uPA as well as ATF on osteosarcoma cells. Also, Fischer *et al*. (1998) demonstrated that HMW-uPA, ATF, and a uPA-related peptide encompassing the

uPAR binding region (cyclo^{19,31}uPA$_{19-31}$) stimulated cell proliferation in the ovarian cancer cell line OV-MZ-6. The mitogenic effect of ATF and of the uPA-derived peptide was observed only in uPA-antisense transfected cells. However in other studies on the mitogenic effect of uPA on human epidermal, melanoma, breast carcinoma and vascular smooth muscle cells, both the enzymatic activity of uPA and its binding to uPAR were required in order to induce a mitogenic response (reviewed by Reuning *et al.*, 1998), and addition of ATF even reduced cell proliferation (Luparello and Del Rosso, 1996). In our case, expression of multifunctional inhibitors containing uPA$_{19-31}$ did not affect cell proliferation *in vitro*.

TIMP-1 was shown to be the homologoue of an erythroid potentiating activity (EPA) factor that stimulates the growth of erythroid precursor cells *in vitro* (Gasson *et al.*, 1985; Avalos *et al.*, 1988). There are also reports on a growth factor-like and anti-apoptotic activity of TIMP-1 on a wide range of non-erythroid cell lines (Bertaux *et al.*, 1991; Hayakawa *et al.*, 1992; Yamashita *et al.*, 1996; Guedez *et al.*, 1998). Since these biological activities are independent of the MMP-inhibitory activity and located at the C-terminus of TIMP-1 (Chesler *et al.*, 1995; Gomez *et al.*, 1997; Brew *at al.*, 2000; Stetler-Stevenson, 2001), N-TIMP-1, which we used for construction of the multifunctional inhibitors, does not harbor any tumor promoting property. Recent studies have shown that the overexpression of TIMP-3 induces the apoptotic cell death of a number of cancer cell lines (Ahonen *et al.*, 1998; Baker *et al.*, 1999; Valente, 1998; Brew *et al.*, 2000). Similar to the growth promoting activity of TIMP-1, the apoptotic feature of TIMP-3 is independent of MMP-inhibitory activity (Bian *et al.*, 1996; Baker *et al.*, 1998, 1999).

Recently, intimate links between the uPA system and the integrin cell adhesive system have been described (Preissner *et al.*, 2000; Chapman *et al.*, 2001; Reuning *et al.*, 2003). Integrins are heterodimeric, transmembrane receptors, consisting of two non-covalently linked α and β subunits. Integrins are expressed on the surface of various cell types and interact with ECM proteins such as vitronectin, fibronectin, collagen, fibrin and laminin *via* the integrin recognition RGD-motif (Giancotti and Rouslahti, 1999, Schwartz, 2001). Several lines of experimental evidence (co-immunolocalization, resonance energy transfer studies) have indicated a physical association between the GPI-anchored uPAR and some members of the integrin subfamilies like β1, β2, and β3 integrins, resulting in altered integrin adhesive functions (Simon *et al.*, 1996; Xue *et al.*, 1997; Wei *et al.*, 2001).

The adhesive behavior of the various transfected OV-MZ-6#8 cell lines was tested on fibronectin (Fn), vitronectin (Vn), and collagen (Col) type IV. For OV-N-hTIMP-1-chCysWT, OV-N-hTIMP-3-chCysWT, and OV-N-hTIMP-3-chCys-uPA$_{19-31}$ cells, binding to

all tested ECM proteins was not altered when compared with vector transfected control cells. In the case of OV-N-hTIMP-1-chCys-uPA$_{19-31}$ cells, a slightly enhanced adhesion to vitronectin, fibronectin, and collagen type IV was observed.

It has been demonstrated that interaction of GPI-uPAR with αvβ3 integrin is essential for uPAR-dependent adhesion to vitronectin and disruption of the uPAR/integrin complexes by uPAR-binding peptides (p25, M25, and α325) blocks adhesion to vitronectin (Wei *et al.*, 1996, 1999, 2001; Simon *et al.*, 2000; Pluijm *et al.*, 2001). uPA and ATF have been shown to enhance uPAR/vitronectin binding affinity and, by this, increase cell adhesion to Vn (Wei *et al.*, 1994, 2001; Kanse *et al.*, 1996; Stahl and Mueller, 1997; Chang *et al* 1998; Yebra *et al.*, 1999). It is possible that the interaction of the uPAR binding site-harboring trifunctional inhibitor, with uPAR will also enhance uPAR/vitronectin binding affinity and by this promote cell adhesion. Influence of uPA/uPAR on integrin-dependent cell binding to Fn and Col seems to be more complicated. On one side, binding of uPA to uPAR had no effect on adhesion of LNCaP cells and myogenic cells to fibronectin or collagen (Planus *et al.*, 1997; Yebra *et al.*, 1999), and overexpression of GPI-uPAR on the cell surface or addition of suPAR resulted in a significant decrease of cell binding to fibronectin and collagen. Addition of p25, which specifically disrupts uPAR/integrin interaction, restored adhesion to Fn (Wei *et al.*, 1996; Pluijm *et al.*, 2001). On the other side, in some cells it has been observed that endogenously expressed uPAR promotes integrin function. Aguirre Ghiso *et al.* (1999) demonstrated that a functional interaction between uPAR and integrins in tumorigenic T-HEp3 cells is necessary for their optimal adhesion to Fn. Furthermore, it was shown that the addition of recombinant uPA enhances spreading of MDA-MB-231 cells on Fn and Col (Wei *et al.*, 2001). In another study it has been demonstrated that the association of uPAR with β1 stabilizes the β1-integrin/caveolin complex and activates a cascade of events leading to an organization of the cytoskeleton, cell adhesion and spreading (Wei *et al.*, 1999). Another mode of action for the trifunctional inhibitor may consist in the disruption of the uPAR/integrin interaction that, as it was demonstrated in previous studies for synthetic peptide M25 and p25 (Wei *et al.*, 1996; Simon *et al.*, 2000; Pluijm *et al.*, 2001), could lead to an enhanced adhesion to fibrinogen and collagen. At the same time it should diminish cell adhesion to vitronectin, as did peptides p25, M25 and an antibody directed against uPAR (Wei *et al.*, 1996, 1999; Simon *et al.*, 2000; Pluijm *et al.*, 2001). Nevertheless, the observed slight increase in the adhesive capacities of OV-N-hTIMP-1-chCys-uPA$_{19-31}$ cells to Fn and Col might be rather a clonal effect than a result from the interaction of the recombinant uPA$_{19-31}$-harboring inhibitor with cell surface uPAR.

Cell invasion is a very complex process that implicates both cell locomotion and active penetration of the cells into the ECM. A variety of cell culture invasion assays are available to explore tumor cell invasiveness, including assays with invasion through an urinary bladder wall, isolated human amniotic membranes, chicken chorioallantoic membranes, blood vessels and assays with invasion of cells into reconstituted ECM, the so-called Matrigel, as well as individual ECM components like fibrin and collagen gels (Andreasen *et al.*, 2000; Horino *et al.*, 2001). The capacity of the multifunctional inhibitors to inhibit ovarian cancer cell invasion was analyzed in *in vitro* Matrigel invasion assays. Both approaches: i) use of transfected cancer cells endogenously producing and releasing the inhibitors and ii) exogenous addition of the recombinant proteins, demonstrated that the multifunctional inhibitors do considerably reduce invasion of ovarian cancer cells. Degradation of the ECM and basement membranes is a crucial first step in the mechanism of tumor cell invasion. Although a variety of protease families are implicated in this process, especially MMPs and members of the serine protease family, such as plasmin and uPA have been the focus of much attention. Overexpression of MMPs have been shown to correlate with a more invasive phenotype of cancer cells since transfection of several cell lines with genes, encoding MMPs caused an increase in tumor cell invasion and migration (Ray and Stetler-Stevenson, 1994; Deryugina *et al.*, 1997; John and Tuszynski, 2001). Recent studies have proposed an additional role of MMPs in modulation of cell attachment and migration. Thus, Lochter *et al.* (1997) reported that E-cadherin function can be interrupted by degradation of E-cadherin's extracellular domains by MMP-3. Also MMP-9 was found to participate in the cleavage and release from the cell surface of the cell-matrix adhesion receptor CD44 (Okamoto *et al.*, 1999). This alteration of cell-cell and cell-matrix adhesion can facilitate cell migration and invasion. Inhibition of cancer cell invasion has been achieved by recombinant TIMPs or by overexpression of TIMPs using a variety of gene delivery vehicles. Overexpression of TIMP-1 in gastric cancer cells reduced metastasis of clones expressing high levels of TIMP-1 in chicken embryos. Recombinant TIMP-1 inhibited *in vitro* invasion of astrocytoma cells, prostate cancer cells and mammary carcinoma cells (Johansson *et al.*, 2000; Stetler-Stevenson and Yu, 2001, Tachibana *et al.*, 2002). Overexpression of TIMP-1 and TIMP-3 significantly inhibited invasion of HeLa and HT 1080 cells through reconstituted basement membranes (Baker *et al.*, 1999). uPA participates in cell-surface associated activation of plasminogen to plasmin, which catalyzes the breakdown of the majority of the ECM molecules and is capable of activating several members of the MMP family. The binding of uPA to its receptor also activates intracellular signaling systems that regulate cytoskeleton rearrangement and

subsequently cell adhesion and migration. Several different *in vitro* assays indicated that the invasive capacities of tumor cells can be down-regulated by inhibition of uPA enzymatic activity, transfection of the cells with anti-sense sequences for uPA or uPAR, inhibition of uPA/uPAR interaction with anti-uPAR antibodies, anti-uPA antibodies or other specific uPA/uPAR-binding antagonists or by 'scavenging' uPA using soluble uPAR (reviewed by Andreasen *et al.*, 1997; Schmitt *et al.*, 2000; Reuning *et al.*, 2003a). Recent studies have shown that cathepsins B, H and L are involved in cancer progression either by direct degradation of extracellular matrix (type I and IV collagen, laminin) or by activation of other proteases. Studies using inhibitors of cathepsins, natural inhibitors such as cystatins as well as synthetic inhibitors, demonstrated their capacity to reduce tumor cell invasion *in vitro* (Corticchiato *et al.*, 1992; Coulibaly *et al.*, 1999; Premzl *et al.*, 2001; Sever *et al.*, 2002; Colella *et al.*, 2002).

Since multifunctional inhibitors effectively reduced invasion of ovarian cancer cells in *in vitro* invasion assay, the effect of the endogenously produced inhibitors on primary tumor growth and spread of human ovarian cancer cells was further analyzed in a *xenograft nude* mouse model. Expression of these recombinant proteins, consisting of N-TIMP-1 or N-TIMP-3 fused to wild type chicken cystatin or chicken cystatin including the uPAR binding site of uPA, resulted in a reduced tumor growth of human ovarian cancer cells. A considerable number of studies has shown that overexpression of TIMPs by tumor cells or host tissues reduces tumor progression and metastasis (summarized in Brand, 2002). Indeed, intraperitoneal injection of recombinant TIMP-1 has been shown to inhibit lung colonization of B16 melanoma cell and overexpression of TIMP-1 inhibits tumor growth and metastasis of melanoma (Yu *et al.*, 1997; Jiang *et al.*, 2002). Adeno- or retroviral delivery of the TIMP-3 gene to human melanoma, SCC and neuroblastoma cells inhibited tumorigenesis after subcutaneous injection of the infected cell into SCID mice (Ahonen *et al.*, 2002; Spurbeck *et al.*, 2002) and overexpression of TIMP-3 in human colon carcinoma cells inhibited tumor growth in nude mice (Bian *et al.*, 1996). There are several mechanisms that may attribute to the anti-tumor effect of TIMPs. First, inhibition of MMPs activity protects ECM and basement membranes from proteolytic degradation, restricting the invasion of tumor cells. Second, TIMPs have anti-angiogenic activity either by direct effect on endothelial cell proliferation or by their ability to inhibit the activity of MMPs required for endothelial cell migration and invasion (Pepper, 2001). A third mechanism based on the fact that extracellular proteolysis has been implicated in the regulation of cytokine activity. Thus, inhibiting MMPs, TIMPs prevent activation of latent transforming growth factor-β, release of matrix-bound bFGF and VEGF,

and release of membrane anchored tumor necrosis factor-α (Hornebeck *et al*., 2002; Pupa *et al*., 2002). However, an increased expression of TIMP-1 was found in a more tumorigenic colon cancer cell line, which is in line with the observation that high TIMP-1 expression is associated with poor prognosis in colorectal cancer (Hewitt *et al*., 2000). In addition, TIMP-1-induced stimulatory effect is also reported in intestinal tumor model in TIMP-1 transgenic mice (Goss *et al*., 1998). This may be related to growth factor-like effects described for TIMP-1 on a wide range of cell lines (Hayakawa *et al*., 1992, Yamashita *et al*., 1996). Although a positive correlation between tumor invasiveness and secretion of cysteine protease (particularly cathepsins B, H and L) has been well documented in literature and a number of *in vitro* studies demonstrated the inhibitory effect of cystatin C on tumor cell invasion, there is only one report on effect of cystatin C on tumor spread *in vivo*. In this study overexpression of human cystatin C by melanoma cells led to a significant reduction of lung colonization after tail vein injection in mice (Cox *et al*., 1999). Many experiments with animal model systems have shown convincingly that u-PA-catalyzed plasmin generation is an important determinant of the rate of the overall process of tumor metastasis. Nevertheless, the binding of uPA to uPAR does not only initiate a cascade of proteolytic events, leading to extracellular matrix breakdown, release of latent forms of growth factors from ECM, and activation of other proteases. This interaction also activates intracellular signaling systems that regulate cell proliferation, adhesion and migration. In view of the important role of uPA system in tumor growth and invasion, numerous approaches have been used to target uPA/uPAR interaction for cancer therapy (reviewed in Andreasen *et al*., 1997; Reuning *et al*., 2003a). In previous studies, a significant anti-tumorigenic effect was achieved also in the OV-MZ-6#8 *xenograft* mouse model by blocking uPA/uPAR interaction with synthetic uPA-derived cyclic peptides (Sato *et al*., 2002) or soluble uPAR (Lutz *et al*., 2001) as a scavenger for uPA. This illustrates the important role of this protease system in the used tumor model. In our study bi- and trifunctional inhibitors were similarly potent in reduction of tumor burden. Since we have previously shown that all components of the multifunctional inhibitors are able to bind their counterparts, it seems that in this tumor model inhibition of MMPs and/or cysteine proteases by the bifunctional inhibitors is already very efficient to reduce the tumor mass. Thus, the introduction of another inhibitory function, the uPA-derived peptide that interrupts uPA/uPAR interaction, yielding trifunctional inhibitors does not result in a further reduction of tumor growth and spread.

Numerous therapeutic approaches have been used to target components of protease systems in cancer cells and tissue (Cox *et al.*, 1999; Muehlenweg *et al.*, 2001; Brand, 2002; Cousens *et*

al., 2002). The functions of the various proteolytic systems may overlap or even be redundant, a lesson learnt from the apparent phenotype of knockout mice deleted for these proteolytic factors (Carmeliet and Collen, 1998). Therefore, the application of multifunctional inhibitors is expected to be more efficient as compared to therapeutic molecules with inhibitory activity against a single system only.

Expression of the multifunctional inhibitors in *E. coli* resulted in a relatively low yield of active protein after purification and refolding. Therefore, the approach of exogenously applying recombinantly produced, purified inhibitors as therapeutic molecules in cancer treatment seems to be rather unattractive. However, the endogenous (relatively low) expression of the inhibitors by transfected ovarian cancer cells showed a dramatic effect on *in vitro* invasion and tumor growth and dissemination *in vivo,* indicating that a therapeutic approach employing *e.g.* viral vectors or other gene transfer systems may be feasible to affect tumor cell growth and spread. The very small size of these compact therapeutic molecules carrying up to three different inhibitory functions may prove beneficial for generating appropriate vectors, especially, if *e.g.* cell type-specific expression should be aspired, where rather large regulatory promoter sequences are used. Finally, one has also to consider that the introduction of any new inhibitory agent in cancer therapy may be easier to achieve with only one therapeutic molecule carrying multiple functions than with *e.g.* three different inhibitor molecules.

6. References

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7. Acknowledgments

These doctoral thesis was performed between the years 2001 and 2003 in the Clinical Research Unit of the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universität München.

I wish to express my grateful thank to Prof. Dr. Manfred Schmitt, head of the Clinical Research Unit of the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, TU München, who generously gave me valuable time and advices correcting my papers and this doctoral thesis.

I would like to extend my warmest thank to PD Dr. Viktor Magdolen, my supervisor, who introduced me to this field and generously gave me advice and suggestions. I also would like to express my gratitude for his spending of valuable time for weekly discussions and correcting my papers and this doctor thesis.

Likewise, I am grateful to Dr. Ulla Magdolen, who was also involved in this project and experiments, and with whom it was a pleasure to work with.

I am grateful to PD Dr. Ute Reuning, who introduced me to cell culture methods and generously gave me valuable time and advices.

My grateful thank to PD Dr. Achim Krüger and Dipl. Biol. Charlotte Kopitz from the Institut für Experimentelle Onkologie und Therapieforschung, TU München, who helped me with the performance of animal experiments.

I am gratefull to Dr. Irmgard Assfalg-Machleidt from the Adolf-Butenandt Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie, LMU München and Dr. Eva-Kathrin Sinner from the Max-Planck-Institut für Biochemie, Martinsried, München, who gave me the possibility to perform experiments using their BIAcore 2000 system.

I am grateful to our technical assistants: Sabine Creutzburg, Cristel Schnelldorfer and Anke Benge, who showed me all of the methods and techniques necessary to realize this project.

I thank the medical and PhD students Elke Guthaus, Florian Schroeck, Angela Kirschenhofer, Sumito Sato and Tomas Langerholc, who supported a good atmosphere of work and made the time, that I stayed in the laboratory, very pleasant.

Finally, I would like to dedicate this work to my husband, who always listened to me and who was a constant source of moral support, as well as to my parents, who continuously encouraged me and supported me throughout my time of education.

8. Curriculum vitae and Publications

Curriculum vitae

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06/1998 - 08/1998	Catholic University, Lille, France, Hospital St. Philibert, medical training at the faculty of Obstetrics and Gynecology
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Oral presentations

2nd Conference on Experimental Tumor Biology, Slovenia. Inhibitors of tumor-associated proteolytic systems. 16.03.02

8. Jahrestagung des Arbeitskreises für Molekularbiologie in der Frauenheilkunde, Wotersen. Multifunctional inhibitors of tumor-associated proteolytic systems. 06.04.02

16th International Congress of the International Society for Fibrinolysis and Proteolysis, München. Trifunctional inhibitors of tumor-associated proteolytic systems. 08.09.02