Cellular Uptake of Cystatin C. Subcellular localisation and intracellular effects of a secreted cysteine protease inhibitor

Wallin, Hanna

2013

Link to publication

Citation for published version (APA):
Wallin, H. (2013). Cellular Uptake of Cystatin C. Subcellular localisation and intracellular effects of a secreted cysteine protease inhibitor Division of Clinical Chemistry and Pharmacology, Faculty of Medicine, Lund University
Cellular Uptake of Cystatin C

Subcellular localisation and intracellular effects
of a secreted cysteine protease inhibitor

Hanna Wallin

DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Segerfalksalen, BMC, Lund
Sept 13 2013 at 13.15

Faculty opponent
Prof. Elvar Theodorsson
Department of Clinical and Experimental Medicine
Division of Clinical Chemistry
Linköping University
Abstract
Cystatin C is a cysteine protease inhibitor, aimed for secretion, as it is produced with a signal peptide. Its target enzymes are thought to be the lysosomal cysteine cathepsins and legumain. Cystatin C has been considered to exert its enzyme inhibiting functions extracellularly, as a defense against enzymes from leaking lysosomes or invading pathogens.

It was demonstrated by various techniques, including flow cytometry, confocal microscopy, ELISA and Western blotting, that cystatin C was internalised in cells of different cell lines after incubation with a physiological concentration of cystatin C. The internalised cystatin C was found in acidic endolysosomal vesicles and co-located with some potential target enzymes, in contrast to the endogenously produced inhibitor, which was mainly found in the endoplasmic reticulum. Cystatin C was non-degraded and still functional as an inhibitor of cysteine cathepsins after uptake, as the total enzyme inhibiting capacity of the cell lysates was increased, suggesting that intracellular cysteine protease activity can be regulated by the uptake. Invasion and migration of MCF-7 breast cancer cells were inhibited when cells were incubated in medium containing cystatin C.

To pin-point the structural requirements for cellular uptake, twelve variants of cystatin C, including wild-type, were produced by site-directed mutagenesis and cleaving of the N-terminal. Positively charged amino acid residues on the surface of the molecule, and the amino acid at position 106 were shown to be important for internalisation. In most cases the uptake was decreased after molecular engineering, but for the variant W106F-cystatin C it was increased. The substitution of W106 affects the cathepsin-inhibiting properties of cystatin C, but it is still an efficient inhibitor of legumain. The increased uptake of this variant also induced an increased inhibition of legumain in lysates of cells after uptake.

Key words
cathepsin, cell line, co-localisation, cystatin C, internalisation, legumain
Cellular Uptake of Cystatin C

Subcellular localisation and intracellular effects
of a secreted cysteine protease inhibitor

Hanna Wallin
Department of Laboratory Medicine
Division of Clinical Chemistry
Lund University

Lund 2013
Cover: Uptake of AlexaFluor488-labelled cystatin C (green) in MCF-7 breast cancer cells, visualised by confocal microscopy. Nuclei stained with DAPI (blue). Image by Bo Holmqvist, ImeGene-iT and Hanna Wallin.
To my family
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEW-cystatin</td>
<td>Chicken egg-white cystatin</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>E64</td>
<td>trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane, an irreversible, potent, and highly selective inhibitor of cysteine cathepsins</td>
</tr>
<tr>
<td>E64d</td>
<td>(2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester, a cell permeable variant of E64</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HCCAA</td>
<td>Hereditary cystatin C amyloid angiopathy</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LRP</td>
<td>Low-density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>NMec</td>
<td>7-amino-4-methyl-coumarin</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor-activator of nuclear factor κB ligand</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TGF-βRII</td>
<td>TGF-β receptor 2</td>
</tr>
</tbody>
</table>
Contents

Abbreviations 6

Contents 7

Introduction 9

 Proteolytic enzymes 9
 Lysosomal cysteine proteases 10
 Clan CA, family C1 11
 Clan CD, family C13 12
 Regulation of proteolytic enzymes 13
 Protease inhibitors 14
 Cysteine protease inhibitors – cystatins 14
 Type 2 cystatins 16
 Biological functions of cystatin C 18
 Hereditary Cystatin C Amyloid Angiopathy (HCCAA) 18
 Inflammation 19
 Differentiation and proliferation 19
 Gene polymorphisms as risk factors 20
 Cystatin C as a marker for kidney function 20
 Type 2 cystatins in cancer 21
 Strategies for internalisation of extracellular molecules 22

Present study 24

 Aim 24

List of papers 25

Material and methods 26

 Cell lines 26
 Expression of cystatin C variants 27
 Quantification of cystatin C in cell lysates 29
 Fluorescence 29
 Western blotting 32
 Enzyme activity assays 32
 Invasion and migration in Matrigel™ invasion chambers 33
Results and discussion 33
  Uptake of cystatins 33
  Subcellular localisation 37
  Uptake mechanism 38
  Functional consequences of the uptake 40
Overall conclusions 42
Future perspectives 42

Ett nytt sätt att se på cystatin C (populärvetenskaplig sammanfattning) 43
Tackord 45
References 47
Introduction

Proteolytic enzymes

Synthesis and degradation of proteins are fundamental for cellular processes. Proteins of different size and shape have different functions, such as signaling, defense, regulation and transport. The cellular protein content varies in different cells and tissues, with the phase of the cell cycle and maturation grade. The distribution, quantity and function of the cellular proteins are dependent on the environment and naturally these protein characteristics may change when a healthy cell becomes malignant.

The amino acids are the building blocks of the proteins. The 20 naturally occurring amino acids, which all have unique side chains and therefore different properties, are connected to each other with peptide bonds and then folded to globular structures. The primary structure of a protein describes the sequence of its amino acids. In some parts of the protein the sequence of amino acids and the interactions of their side chains can generate a spiral-like structure, an α-helix, or a structure with the amino acid residues arranged side by side like a sheet, a β-sheet. The α-helix and the β-sheet arrangements are referred to as secondary structure. The globular three-dimensional folding is called the tertiary structure and when two or more proteins are connected to form a larger unit it is called the quaternary structure of a protein (Fig. 1). The characteristics of the protein may also be defined by molecules, like carbohydrates or lipids, which can be connected to the amino acid residues.

More than 500 genes, about 2% of the human genome, code for proteins that degrade other proteins by cleaving the peptide bonds between the amino acids (1). These proteins are called proteolytic enzymes, proteases or peptidases and are denominated as exo- or endopeptidases depending on their cleaving properties. Exopeptidases disrupt peptide bonds from the end of the amino acid chain, either from the N-terminal or the C-terminal, while endopeptidases break the bonds within the polypeptide. The active site is where the substrate, i.e. the protein that is going to be cleaved, binds and it contains either an amino acid residue (aspartic acid, cysteine, serine, threonine or glutamine) or a metal ion important for the catalytic mechanism. This is the base for another way of classification and the major classes of proteases are called aspartic, cysteine, serine, and metallo-proteases. In the next level of classification, found in the database MEROPS, the enzymes are divided in clans and families due to their structural relationship (2,3).
Fig. 1 Proteins are folded in different ways according to the amino acid sequence and the interactions of their side chains. Courtesy: National Human Genome Research Institute (NHGRI).

Lysosomal cysteine proteases

Lysosomes were first described by de Duve et al. 1955 (Fig. 2) (4). They are cellular organelles surrounded by a membrane bilayer, which allows a pH-value about 5 and a high content of enzymes with capacity to degrade proteins, nucleic acids, lipids and carbohydrates in the acid environment. Lysosomes are formed by the fusing of vesicles from the endoplasmic reticulum, containing newly produced enzymes, and endosomes, produced by the ingestion of substances from the cell exterior. The endosomes follow the endolysosomal pathway, with a step-wise maturation via early and late endosomes to the mature lysosome.
Clan CA, family C1

Papain from the papaya fruit is the best characterized protease of clan CA, family C1, “the papain-like cysteine proteases” and is used as a model enzyme for studies of the mammalian analogues, the cysteine cathepsins B, C, F, H, K, L1, L2/V, O, S, W and Z/X (Fig. 3) (3). It is worth mentioning that not all cathepsins are cysteine proteases. For example, cathepsin D is an aspartic and cathepsin G is a serine protease. Cathepsins are produced as preproenzymes, cleaved in the endoplasmic reticulum to proenzymes and subsequently transported to the lysosomes. The acidic pH in these organelles allows further processing of the proforms to active mature enzymes. The subcellular localisation of the enzymes might change in a cancer cell, and the enzymes are redirected from their normal lysosomal location to structures associated with the plasma membrane, like caveolae. This can be followed by secretion of the enzymes to the extracellular environment. (5-9). Recently, presence and activity of cathepsins B and L has been shown to be involved in the processing of histones in the nucleus of cancer cells (10-12).

Most of the lysosomal cathepsins are ubiquitously expressed and are traditionally thought of as rather unspecific enzymes, responsible for the normal turnover of proteins. Some of the cathepsins are known to be predominantly expressed in specialised cells, which indicates their involvement in specific processes. For example, cathepsin K is expressed in
osteoclasts and thereby involved in bone remodeling and resorption (13). Expression of cathepsin W in cytotoxic cells (T-cells and NK-cells) and of cathepsin S in the antigen-presenting cells (dendritic cells, macrophages and B-cells) results in a contribution to the defense against pathogens (14-16).

Increased expression of cathepsin B and L has been reported in human tumours of the breast, colon, ovary, brain and prostate and in some studies the level of the enzymes has been related to prognosis of the disease (17-21). Under normal conditions cathepsin B takes part in the protein degradation in the lysosomes, but in cancer, due to the translocation of the enzyme, this protease may be involved in degradation of the extracellular matrix and thus plays an important role in metastasis and invasion of tumour cells. The involvement of cathepsin B in extracellular matrix degradation is either indirect, by activating other enzymes as metalloproteases and urokinase-type proplasminogen, or direct, by degradation of substances of the extracellular matrix such as collagen, laminin, fibronectin and the cell adhesion protein E-cadherin (22-24). In addition, breast cancer cells have been shown to engulf (phagocytose) the extracellular matrix and degrade it in lysosome-like vesicles (25).

Clan CD, family C13

Legumain (asparaginyl endopeptidase) was characterized in mammals as recently as in 1997 (26). It is a lysosomal cysteine protease of clan CD, family C13 unrelated to the cysteine cathepsins (Fig. 3) (3). Like these enzymes, legumain is expressed as an inactive proenzyme and activated as a consequence of the acidic pH of the lysosomes (27). The bond-cleaving specificity of legumain is very restricted; it only cleaves after asparagine residues (26). Besides its contribution to normal lysosomal degradation of macromolecules, legumain is a key enzyme in the dendritic cell antigen processing (28). Up-regulation of legumain is seen in tumours and tumour-associated cells and has been shown to correlate with bad prognosis (29-31). In addition, legumain has an important function in regulation of other enzymes by processing cathepsins B and L and activating pro-matrix metalloprotease-2 (32,33)
Regulation of proteolytic enzymes

The function of a proteolytic enzyme, a protease, is to catalyse the hydrolysis of a peptide bond by binding a substrate to its active site (like a key in a keyhole) and diminish the activating energy that is needed to perform the hydrolysis (Fig. 4). The substrate concentration is central to the velocity of the reaction i.e., the more substrate, the faster reaction until the maximum rate is reached. The Michaelis constant, $K_m$, describes the affinity between enzyme and substrate and is defined as the substrate concentration when the reaction proceeds at 50% of the maximal speed. A low $K_m$ means that the binding of substrate to enzyme is tight and that the reaction will reach its maximum at a low concentration of substrate. A high $K_m$ means that the affinity for the substrate is low and that high concentration of substrate is needed to reach the maximum rate of the reaction.

Many enzymes are produced as inactive zymogens and activated at the site where they are supposed to execute their functions. One example of this is inactive trypsinogen that is secreted from the pancreas and then cleaved to active trypsin by the enzyme enterokinase in the small intestine. The pH in the compartment can affect the catalytic site or the enzyme conformation which results in enzyme activation, like in the lysosomes. Changes of gene expression or degradation of the enzyme as a consequence of extracellular signals and environmental factors have an impact on enzyme regulation due to variations of the concentration of active enzyme.
Protease inhibitors

The proteases are also regulated by inhibitors, which are molecules that either bind to the enzymes’ active site and thus prevent cleaving of the substrate or to another part of the enzyme, thereby changing the conformation of the molecule. When the inhibitor competes with the substrate to bind to the active site it is called competitive inhibition (Fig. 4), while the situation when the inhibitor binds to another part of the enzyme is called non-competitive (or allosteric) inhibition. Binding of the inhibitor to the enzyme can be either reversible or irreversible. Specificity of the inhibitors is fundamental for the interplay between the inhibitors and the enzymes. Some of the inhibitors, like α-2-macroglobulin, are non-specific, which means that they inhibit enzymes from all classes. Other inhibitors are specialised to act on enzymes of specific catalytic classes, like serine protease inhibitors (serpins) and cysteine protease inhibitors (cystatins).

![Diagram of enzyme inhibition](image)

**Fig. 4** Competitive enzyme inhibition. The substrate fits into the active site of an enzyme in order to be cleaved. The resulting molecules are called products. Competitive inhibitors, like the cystatins, compete with the substrate of docking into the enzymes’ active site, thereby preventing cleaving of the substrate. E=enzyme, S=substrate, P=product, I=inhibitor.

Cysteine protease inhibitors – cystatins

The human cystatin family is constituted by three types (Fig. 5). The intracellular type 1 inhibitors cystatin A and B (also called stefin A and B) belong to subfamily I25A according to MEROPS classification (3). They are small proteins (∼100 amino acids, 11
kDa), without disulfide bonds and carbohydrate side-chains, found in the cytoplasm of most cells. Besides being an intracellular inhibitor, cystatin A may be found in body fluids, like urine and milk (34,35).

The extracellular type 2 cystatins, which include cystatin C, D, E/M, F, G, S, SA and SN, are found in MEROPS subfamily I25B (3). These inhibitors are larger than the type 1 cystatins (~120 amino acids, 13 kDa) and contain in the normal case two disulfide bridges (three for cystatin F). In contrast to other type 2 cystatins, cystatins E/M and F are special, as they can be glycosylated (35).

Finally, the kininogens (high-molecular-weight-kininogen and low-molecular-weight-kininogen) are referred to as type 3 inhibitors. They are the main inhibitors in blood and synovial fluid (36). The kininogens contain three repeated cystatin C domains, of which two are active, and a kinin precursor domain. As a consequence of this, they belong to the MEROPS subfamily I25B as the type 2 cystatins, because each domain is seen as an individual inhibitor unit (3).

**Fig 5.** Human cystatins. The intracellular type 1 cystatins are synthesised without disulfide bonds. The extracellular type 2 cystatins contain two disulfide bonds (exception: cystatin F with three disulfide bonds). The type 3 cystatins, the kininogens, contain three repeated type 2 domains. Cystatins are normally not glycosylated, but exceptions are cystatins E/M and F. I will in this thesis focus on type 2 cystatins, mainly cystatin C.

Chicken cystatin was the first cystatin structure to be revealed. All cystatin structures described since then have similarities in their sequences which give them a certain
"cystatin fold" (Fig. 6). This implicates a five-stranded β-sheet wrapped around an α-helix (37) and conserved Gln-Xaa-Val-Xaa-Gly and Pro-Trp segments at positions 55-59 and 105-106, respectively (cystatin C numbering). Together with some amino acids of the N-terminal segment (positions 8-11) these conserved sequences constitute the cysteine protease binding site (38). The protease inhibition is achieved through binding of the inhibitor to the substrate-binding pocket of the enzyme (39).

**Fig. 6** Cystatin fold. Cystatin structure contains an α-helix (yellow) surrounded by a five-stranded β-sheet (blue). The cathepsin-binding site is formed by amino acid residues near the N-terminal and in the first and second loops. The residue at position 39, asparagine, is considered to be important for legumain binding. Adopted from Alvarez-Fernandez, M., Liang, Y. H., Abrahamson, M., and Su, X. D. (2005) Crystal structure of human cystatin D, a cysteine peptidase with restricted inhibition profile. *J. Biol. Chem.* 280, 18221-18228

**Type 2 cystatins**

The inhibitor constant K, shows how powerful the inhibitor is to inhibit its target protease, as the value represents the concentration needed to achieve half maximum inhibition of the enzyme. The type 2 cystatins are generally potent inhibitors of all papain-like cysteine proteases with K, values between $10^{-7}$ and $10^{-12}$ M. The interaction between cystatin C and papain is extremely good with K, about $10^{-14}$ M (40). Inhibition
of legumain is accomplished only by three type 2 cystatins, cystatin E/M (K_i=0.0016 nM), cystatin C (K_i=0.20 nM) and cystatin F (K_i=10 nM) (41). Many of the type 2 cystatins have low affinity for cathepsin B as the active site of the enzyme partially is blocked by an occluding loop, which has to be removed before the inhibitor can bind to the enzyme (42). Cystatin C is the most effective inhibitor of cathepsin B with a K_i value of 0.22 nM. The inhibition is performed in a two-step reaction. An initial, weak interaction between the N-terminal region of cystatin C and the protease is followed by a conformational change leading to a dislocation of the occluding loop (43).

Cystatin C is the most potent and well studied of the type 2 inhibitors. It is expressed by most cells and can be found in all body fluids, with the highest concentrations in seminal plasma (3.7 µM) and cerebrospinal fluid (0.5 µM) (34,44). The mature inhibitor contains 120 amino acids, but cystatin C is produced with a signal-peptide sequence, normally 26 amino acids long, which is cleaved off before the molecule is transported out of the cell.

The enzyme-binding site of cystatin C correlates to amino acid residues Arg8-Leu9-Val10-Gly11 near the N-terminal domain, Gln55-Ile56-Val57-Ala58-Gly59 in the first hairpin loop and Pro105-Trp106 in the second hairpin loop (Fig. 6). Cystatin C, like all other cystatins, binds the enzyme in a 1:1 complex. The cystatin C molecule is a multi-inhibitor able of binding a legumain molecule at the same time as a cathepsin molecule (in this case it is a 1:1:1 complex). This is possible because Asp39, the key amino acid residue for inhibition of legumain, is situated on the opposite side of the cystatin C molecule, compared to the cathepsin-binding site (Fig. 6) (41).

Cystatin E/M was first described as the product of a down-regulated gene in metastasis of breast cancer (45) and as an expressed sequence tag in amniotic membrane cell and fetal skin epithelial cell libraries (46). It was shown to have about 35% sequence identity to cystatin C and contained conserved amino acid residues important for cysteine cathepsin inhibition. Cystatin E/M was later on revealed as the best inhibitor of legumain, due to a second binding site at the opposite side of the molecule (N39) (Fig. 6) (41). It is considered to be a tumour suppressor with down-regulated expression in gliomas (47), cancer of the prostate (48) and gastric carcinomas (49). Additionally, up-regulation of cystatin E/M results in altered motility and metastatic properties of melanoma cells (50).

Cystatin F is highly expressed in immune cells (51,52), which is the reason for its alternative name, leukocystatin. It has been reported that the molecule is secreted as an inactive dimer and subsequently cleaved to an active monomer in endolysosomal compartments (53-55). Even though the inhibitor is transported out of the cell, the intracellular concentration is high in relation to cystatin C (56). Since cystatin F is expressed mainly by immune cells, it opens up for specialised functions in regulation of processes related to immunological response. Cathepsin S, which is involved in antigen presentation, has been suggested to be a target enzyme for cystatin F (56,57).
Very little is known about the cystatins D, S, SA and SN, sometimes referred to as glandular cystatins or salivary cystatins as they are mainly found in saliva (34,58-60), and in tears. The S-like cystatins are additionally present in urine and seminal plasma. A hypothesis is that the function of these cystatins is to maintain oral health (61). Interestingly, cystatin D seems to be a tumour suppressor in colon cancer, regulated by vitamin D (62). Some reports also suggest that up-regulation of cystatin SN expression is involved in cancers of the gastrointestinal tract (63,64).

**Biological functions of cystatin C**

Cystatin C, originally called γ-trace, was first found as a band in the γ-zone of the electrophoretogram, when proteins of cerebrospinal fluid and urine were separated (65-67). In 1982 the amino acid sequence of γ-trace was revealed by Grubb and Löfberg (68) and in 1984 it was found to be a cystatin (69). The name cystatin C was introduced the same year by Barrett *et al.* (70). Immunohistochemistry demonstrated localisation of cystatin C in secretory and neuroendocrine cells of the pituitary gland, adrenal medulla, pancreatic islets and brain cortical neurons, which indicated that cystatin C was involved in the neuroendocrine system (68,71-73).

The exact biological functions of cystatin C has not yet been revealed but the main physiological role is traditionally believed to involve regulation of secreted cysteine cathepsins from leaking lysosomes of disrupted cells and/or invading pathogens. Pathological conditions, such as rheumatoid arthritis, atherosclerosis, Alzheimer’s disease and malignancy, generally include increased protease activity (74-79). Membrane associated and secreted enzymes from cancer cells are capable of degrading the components of the extracellular matrix, which is important for tumour growth, invasion and metastasis as well as angiogenesis (8,80,81). Given this, cystatin C is thought to be very important to maintain the proteolytic balance in pathological conditions and in the cancerous tissue.

**Hereditary Cystatin C Amyloid Angiopathy (HCCAA)**

It was early noted that in some Icelandic families many of the family members were suffering from repeated haemorrhages in early life and most often died before 40 years of age (82). Later on immunohistochemistry of brain tissue from HCCAA patients showed that insoluble plaques of amyloid were localised in the smooth muscle cells of the small vessels (83-85). It was revealed that the main component of the amyloid was a variant of cystatin C lacking the first 10 amino acids of the N-terminal part and with an amino acid substitution of glutamine at position 68 for leucine (L68Q-cystatin C), caused by a single base mutation in the cystatin C gene (83,86).
Wild-type cystatin C is highly stable to both temperature and pH-changes (38, 87). Even so, with time it starts to dimerize by so called domain-swapping (88-91). Due to the amino acid substitution, L68Q-cystatin C is much more unstable and starts to dimerize spontaneously already at 25°C (92). All patients with HCCAA are heterozygous for the mutation and thus express both wild-type and L68Q-cystatin C.

To find out if there was a difference in cystatin C secretion and/or intracellular content in cells from individuals with the L68Q-mutation and healthy controls, monocytes were isolated and cultured. Cystatin C secretion was measured in the culture medium and intracellular content was measured in cell lysates. The results showed that the secretion of cystatin C was lower from the cells with the mutation compared to the healthy controls, which could be an effect of intracellular retention of the L68Q-cystatin C (93). This was further studied in NIH/3T3 cells transfected with plasmids containing wild-type or L68Q-cystatin C genes. A difference in cellular transport and secretion was confirmed as the secretion of L68Q-cystatin C was comparatively low and it was also shown that the mutant was accumulated in the endoplasmic reticulum (94, 95).

**Inflammation**

At sites of inflammation the balance between proteases and inhibitors, including cathepsins and cystatin C is disturbed, with enhanced enzyme activity and/or decreased inhibition (76). Inflammatory cells, like monocytes, macrophages, T-cells and dendritic cells, infiltrate the inflamed tissues, some of them with high production of cystatin C (96).

The impact of cystatin C on inflammatory reactions has been demonstrated by incubating interferon-γ activated murine peritoneal macrophages with cystatins (97-99). This lead to both up-regulated levels of inducible nitric oxide synthase mRNA and increased release of nitric oxide, an important player in regulation of the immune response. Enhanced levels of interleukine-10 and tumour necrosis factor-α could also be detected.

Even though cystatin C is highly produced by inflammatory cells, the influence on the plasma cystatin C level is limited. No correlation was found when the cystatin C plasma level was measured in patients after surgery and compared to the plasma levels of common inflammatory markers (C-reactive protein (CRP), serum amyloid A, haptoglobin and orosomucoid) (100). However, other authors have reported of cystatin C correlation with the inflammatory protein CRP (101, 102).

**Differentiation and proliferation**

Cystatin C has been proposed to have effects on cell differentiation and proliferation. About 20 years ago it was shown that incubation of murine fibroblastic NIH/3T3-cells
with chicken cystatin stimulated proliferation (103) and that rat cystatin C had the same effect on rat mesangial cells (104). It was also shown that a glycosylated form of cystatin C was needed as a co-factor for fibroblast growth factor-2 activity on neuronal stem cell proliferation and differentiation (105). The human cystatin C has normally not a site for N-linked glycosylation, nevertheless the same effects on neurogenesis and astrocytogenesis has been seen (106).

To keep the skeleton intact a balance between the bone-forming osteoblasts and the bone-resorbing osteoclasts must be accomplished. Osteoclasts are formed by fusion of hematopoietic myeloid cells in response of receptor-activator of nuclear factor κB ligand (RANKL) binding to its receptor RANK, a process regulated by cystatin C (107). Cystatin C has been reported to inhibit osteoclast activity as it is an efficient inhibitor of cathepsin K, which is the key cathepsin in bone and cartilage degradation (108,109). Inhibition of osteoclast formation has also been reported in bone marrow and spleen cell cultures incubated with cystatin C and stimulated with parathyroid hormone, vitamin D or interleukin-6 (110).

Gene polymorphisms as risk factors

The cystatin C gene, CST3, is located on chromosome 20p11.2 (111-113). The gene is 4.3 kb and consists of three exons separated by two intron sequences of 2252 and 1254 bp, respectively (44). Upstream of CST3 is the promoter region found, as a 1-kb sequence including two binding sites for the Sp1 transcription factor and an enhancer sequence. Cystatin C mRNA has been demonstrated by Northern blot analyses in all tissues examined (kidney, liver, pancreas, intestine, stomach, antrum, lung, seminal vesicles and placenta) (44).

Cystatin C has been found to co-localise with amyloid plaques in the arterioles in brains of patients with neurodegenerative disease (114,115), but the mechanism is not yet known. A polymorphism was identified in CST3, which resulted in an alanine to threonine substitution of the penultimate amino acid residue of the signal peptide (116). This may affect the normal cleaving site of the protein and result in improper cleaving of the signal peptide, followed by intracellular retention of cystatin C (117). This polymorphism has in some studies been proposed as a risk factor for late-onset Alzheimers disease (118), age-related macular degeneration (119) and cardiovascular disease (120,121), but in other studies no such correlation could be found (122,123).

Cystatin C as a marker for kidney function

Cystatin C is normally filtered freely through the glomeruli in the kidney and reabsorbed by the megalin receptor on epithelial cells in the tubuli for recycling of amino acids (124). The cystatin C production is essentially constant (124,125) and not affected by
gender or age. The plasma level of cystatin C is therefore normally stable and can be used as a marker for glomerular filtration rate. In case of a glomerular injury the filtration rate is disturbed resulting in an elevated plasma level of cystatin C. An increased plasma level can also be seen in pregnancy and serves as an early marker for pre-eclampsia. Filtration through glomeruli is in this case restricted due to endotheliosis (swelling of endothelial cells) (126).

**Type 2 cystatins in cancer**

The role of cystatins in cancer is not clear-cut even though many attempts have been made to delineate the interplay between the inhibitors and the target enzymes and to find out if cathepsin and/or cystatin levels are of prognostic or diagnostic value. Increased cathepsin levels (5,6,127) and/or decreased cystatin C levels are demonstrated both in cancer tissue and in different body fluids. For example, the expression of cystatin C was decreased in relation to the expression of cathepsin B in breast cancer tissue (128), and the level of cathepsin B-cystatin C complex in sera from patients with lung or colorectal cancers was decreased in relation to the level in sera from healthy controls (129). The activities of cathepsins B and H were increased while the concentration of cystatin C was decreased in cerebrospinal fluid from patients with leptomeningeal metastasis (130), and in cyst fluid from ovarian tumours cathepsin B was increased and cystatin C decreased in the most malignant tumours (19).

Effects of cystatin C (and synthetic cysteine protease inhibitors) on cancer cell migration, invasion and motility have been studied in various cell lines. Overexpression of cystatin C in murine squamous carcinoma cells (131), B16F10 melanoma cells (132,133) and human glioblastoma cells (134) as well as addition of inhibitors to cells from human breast (135) and ovary (136) cancer resulted in decreased invasion and growth of the cells.

Cystatin C effects on metastasis have been studied *in vivo* by injecting cystatin C-overexpressing melanoma cells in mouse tail veins. In one study cystatin C was found to reduce lung colonization of the melanoma cells and to increase survival time (137), in another study cystatin C-overexpressing mice had fewer lung colonies of human fibrosarcoma cells (138). Interestingly, tail vein injection of melanoma cells in cystatin C-deficient mice, resulted in fewer and smaller lung colonies of tumour cells compared to the wild-type mice (139).

Cystatin C has been reported to interfere with the surface-located receptor transforming growth factor-β receptor 2 (TGF-βRII) and thereby inhibit the tumour-promoting signaling of transforming growth factor-β (TGF-β) (140,141). TGF-β participates in a variety of biological processes, such as regulation of cell growth and proliferation,
production of extracellular matrix and angiogenesis (142-144). The signaling is mediated through TGF-βRII.

As mentioned earlier cystatin C is able to inhibit legumain, but cystatin E/M has the best capacity for this. Legumain is highly expressed in tumours from breast and in melanoma (29) and cystatin E/M has been shown to reduce the invasive properties of melanoma cells (50). The expression of cystatin E/M in many cancers is reduced or lost due to epigenetic silencing of the gene (47-49,145).

Cystatins D and SN are other type 2 cystatins related to cancer. Cystatin D has been suggested to have tumour suppressing properties induced by vitamin D in colon cancer (62) and cystatin SN has been shown to be upregulated in gastric cancer and thereby contribute to reduction of cell proliferation and cathepsin inhibition (63,64).

Whereas enhanced protease activity often is seen in malignancies, cystatins are important tools for control and regulation of the proteolytic enzymes and the processes they participate in. This may be achieved by 1) extracellular inhibition of secreted enzymes by secreted cystatins, 2) intracellular inhibition of endolysosomal enzymes by endogenously produced cystatins or 3) intracellular inhibition of endolysosomal enzymes by internalised cystatin molecules.

Strategies for internalisation of extracellular molecules

The cellular barrier against the environment is the plasma membrane, which consists of a double layer of phospholipid molecules. The plasma membrane allows passage of small hydrophobic and polar molecules by passive diffusion (e.g. O2, H2O). Larger polar molecules cross the barrier by facilitated diffusion, which means that they are dependent of a transport protein, even though the transport is passive (e.g. transport of glucose). Passive transport is always a consequence of the concentration gradient, from high to low concentration.

All charged molecules need to be transported across the plasma membrane in an active, energy dependent, way. This is also the case when a substance has to be transported against a concentration gradient. A transport protein can be like a pump, like the Na+/K+ pump, where transport of extracellular K+ in to the cell is coupled to transport of intracellular Na+ out of the cell.

Receptor-mediated endocytosis is another example of active transport. The receptor is a protein associated to the plasma membrane with ability to bind a ligand for transport to the cell interior. The cellular response is dependent on the type of ligand and receptor. When the ligand binds to the receptor the plasma membrane starts to invaginate and finally buds off to generate an endosome. The receptors are often recycled back to the plasma membrane while the ligands follow the endosomal pathway to finally fuse with a
primary lysosome. For example, low-density lipoprotein (LDL) is degraded in the lysosome after uptake by the LDL-receptor, while the receptor is recycled (reviewed in (146)). Another example is iron-binding transferrin, which is internalised by the transferrin receptor. After release of the iron in an endosome, both the transferrin and the receptor are recycled back to the plasma membrane (147).

Megalin, also referred to as low-density lipoprotein-related protein 2 (LRP2), on the tubular epithelial cells of the kidney, has been reported to internalise cystatin C for lysosomal degradation (124). Megalin is a multiligand member of the LDL-receptor family. It is expressed on the surface of various epithelial cells, including kidney tubular cells, ciliary epithelial cells of the eye, absorptive intestinal cells, epididymal epithelial cells, cells of the inner ear, and pneumocytes of the lung alveoli (reviewed in (148)).

Enzyme-inhibitor complexes formed in the circulation are rapidly removed by cellular receptors (149,150). This was originally demonstrated by α-2-macroglobulin, which disappeared quickly from the plasma after binding a proteinase (149). The receptor for this was identified as low-density lipoprotein receptor-related protein-1 (LRP1) (alternative name CD91). In addition to α-2-macroglobulin, LRP1 binds a number of ligands, such as lipoprotein particles, serpin-enzyme complexes and proteases. Basic amino acid residues (lysins) have been identified to be crucial for docking of the ligand to the receptor (151). Interaction has been shown between LRP1 and the aspartic protease cathepsin D (152) as well as with cystatin C (personal communication with E. Liaudet-Coopman, Institut de Recherche en Cancérologie de Montpellier, France).
Present study

The balance between proteases and their inhibitors is crucial to maintain the healthy state of a cell. In many diseases the balance is disturbed, with either increased or decreased expression or activity of enzymes or inhibitors. Cystatin C has until recently been considered as an exclusively secreted cysteine-protease inhibitor, which only exerts its effect extracellularly.

The concept of cellular internalisation of cystatin C was formulated as a consequence of experiments in which chicken egg-white cystatin (CEW-cystatin) seemed to have intracellular activity although it had been administered extracellularly. For example, CEW-cystatin added to cell cultures was shown to inhibit replication of polio, herpes simplex and coronavirus (153-155) and added to cultures of murine peritoneal macrophages, it resulted in increased expression and production of nitric oxide and interleukin 10 (98). It was further shown that after injection of cystatin C in cystatin C-deficient mice, it was detected within cells in several organs normally containing endogenous cystatin C (156).

The work that is the base of this thesis concerns intracellular cystatin C and the interesting and exciting questions if, where, how and when the extracellular inhibitor cystatin C meets the lysosomal target enzymes.

Aim

The aim of my work was to

1) clarify whether a system for uptake of cystatins exist
2) investigate the localisation of type 2 cystatins within cancer cells
3) illustrate cystatin C properties important for internalisation
4) describe the functional/biological consequences of the uptake process
List of papers

The thesis is based on the following three papers, which in the text will be referred to by their Roman numbers.


**Summary**: We demonstrated that cystatin C was taken up in a seemingly specific manner by various cancer cell lines in biologically significant quantities, with a final destiny in lysosome-like structures/vesicles. The internalised protein was intact and still functional as an inhibitor of some possible target enzymes (aims 1, 2 and 4).


**Summary**: An unusually high secretion and intracellular content as well as an uptake of cystatin C was demonstrated in neuroblastoma cells. In an earlier study, cells from patients with hereditary cystatin C amyloid angiopathy was seen to have high intracellular cystatin C content. By transfecting SK-N-BE(2) cells with expression vectors for wild-type or L68Q-mutated cystatin C, we could illustrate this special case of elevated cystatin C levels within cells of the neuroendocrine system (aims 1 and 2).


**Summary**: Twelve cystatin C variants (including wild-type) were expressed, purified and added to cultures of MCF-7 breast cancer cells. The uptake of the different cystatin C variants was quantified by ELISA. Charged amino acid residues were found to be important for the internalisation as well as the residue at position 106. W106F-cystatin C was taken up more efficiently than the wild-type inhibitor, but the uptake of the variant W106G was hardly detectable at all. The intracellular inhibition of target enzymes (cysteine cathepsins and legumain) was increased after cystatin C uptake and incubation with cystatin C also decreased invasion and migration properties of the MCF-7 cells in Matrigel. The internalised cystatin C was found in vesicular compartments and co-localised with the lysosomal markers cathepsin D and legumain (aims 1, 2, 3 and 4).
Material and methods

The major methods used in papers I, II and III are briefly described and discussed below. For additional details and complete references to suppliers of reagents used, see the original papers.

Cell lines

The experiments were performed on human cancer cell lines from different origins (Table 1). The cell lines used were either epithelial or neuroendocrine (neuroblastoma). Epithelial cells are applicable as they cover the surfaces of the body and organs, and one aspect of cystatin function is to protect against pathogens invading the body (157). Some cystatins are highly expressed by sweat and salivary glands (cystatins D, S, SA and SN) and cystatin E/M in the epithelial cells of the skin. Neuroendocrine cells have been shown to be heavily stained for cystatin C in various organs, including prostate and brain (158).

Some of the cell lines were chosen for thorough analyses due to their characteristics of secretion and intracellular content of cystatin C, their uptake ability or the capacity for transfection. We started with uptake experiments in five different cell lines (Capan-1, A431, MCF-7, MDA-MB-453, and MDA-MB-468) of which we chose Capan-1 pancreatic carcinoma cells and A431 epidermoid carcinoma cells for detailed studies (paper I). Five neuroblastoma cell lines with different degrees of malignancy were chosen for detailed studies, LA1-5s, SK-N-BE(2), CHP-212, CHP-234, and SH-SY5Y, after initial characterization of intracellular and secreted cystatin C (paper II). MCF-7 breast adenocarcinoma cells were one of the original five cell lines (paper I) and for their good culturing and uptake characteristics they were further used to study cystatin C properties important for uptake and for studies on effects of intracellular cystatin C (paper III). Additionally, three different prostate cell lines, PC3, DU145, and LNCaP, were used to confirm the results obtained with the MCF-7 cells (paper III). MCF-7 cells were also used as a reference cell line (paper II).

All cell experiments were carried out in triplicate and repeated at least twice to ascertain reproducible and reliable results.
Table 1 Cell lines used in uptake studies in papers I, II and III.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capan-1</td>
<td>Epithelial (pancreas)</td>
</tr>
<tr>
<td>A431</td>
<td>Epithelial (epidermis)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Epithelial (breast)</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>Epithelial (breast)</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Epithelial (breast)</td>
</tr>
<tr>
<td>LA1-5s</td>
<td>Neuroblastoma (S-type)</td>
</tr>
<tr>
<td>SK-N-BE(2)</td>
<td>Neuroblastoma (I-type)</td>
</tr>
<tr>
<td>CHP-212</td>
<td>Neuroblastoma (I-type)</td>
</tr>
<tr>
<td>CHP-234</td>
<td>Neuroblastoma (N-type)</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Neuroblastoma (N-type)</td>
</tr>
<tr>
<td>PC3</td>
<td>Epithelial (prostate)</td>
</tr>
<tr>
<td>DU145</td>
<td>Epithelial (prostate)</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Epithelial (prostate)</td>
</tr>
</tbody>
</table>

Expression of cystatin C variants

Recombinant wild-type cystatin C was expressed in *Escherichia coli* (*E. coli*) and purified by ion-exchange and size exclusion chromatographies (papers I and III). An N-terminally truncated variant of cystatin C, lacking the 10 first amino acids, was produced by incubation of wild-type cystatin C with leukocyte elastase, and another 10 variants of the wild-type inhibitor were produced by site-directed mutagenesis followed by *E. coli* expression (paper III). As basic and hydrophobic amino acid residues have been seen to be involved in cellular uptake we chose to replace some of them with glycine or alanine residues. After purification by ion-exchange (Butyl-S Sepharose™ or Q-Sepharose™) and in one case size exclusion (Sephadex™) chromatography the quality of the cystatin C variants was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), mass spectrometry and enzyme activity assays. All variants were found to be at least 90% pure and all variants seemed to be properly folded and functional as inhibitors of the enzymes expected (Fig. 7).

It was crucial to carefully determine the concentration of the added protein in order to be able to accurately compare the intracellular levels of cystatin C after incubation with the different inhibitor variants. Measuring of absorbance at 280 nm is a common method for protein concentration determination. Aromatic amino acids of a protein are responsible for most of the absorption seen at 280 nm, but the structure of the protein can also affect it. The substitution of certain amino acid residues may therefore change the light
absorption properties of the protein. Some of our cystatin C variants lack the only tryptophan residue of the whole molecule, which has a great impact on the absorbance at 280 nm. To calculate the protein concentration in a sample of pure cystatin C the factor 0.83 (both theoretically and practically determined) was normally used. For the variants involving amino acid change of W106 we instead used the factor 0.47, as calculated by ProtParam (http://web.expasy.org/protparam/).

Coomassie Protein Assay is another method to determine the protein concentration. A protein-binding dye that changes colour when it binds to positively charged amino acid residues, is added to the sample before the absorbance is measured and compared to a calibration curve, prepared by dilutions of bovine serum albumin with a known concentration. The amino acids that can be positively charged are arginine, lysine and histidine. Arginine and lysine residues are exchanged in some of the cystatin variants we have produced, but the impact of this is probably minor as the cystatin C molecule contains seven lysine residues and eight arginine residues.

The third method we used to measure the cystatin C concentration of some of the expressed proteins was immunochemical, enzyme-linked immunosorbent assay (ELISA). A good correlation of the results was achieved by the different methods, suggesting that no change of the epitopes recognized by the antibodies used in the ELISA, was introduced by the different amino acid changes of the cystatin C molecule.

---

![Fig. 7](image_url) Analysis of expressed and purified cystatin C variants by SDS-PAGE. Lane 1: molecular size marker. The molecular weight of wild-type cystatin C is 13343 Da. Lane 2: wild-type cystatin C. Lanes 3-13: variants of cystatin C. Des1-10: cystatin C with N-terminal amino acids 1-10 cleaved off. 219/214: (R8G, L9G, V10G, W106G)-cystatin C, 214: (R8G, L9G, V10G)-cystatin C.
Quantification of cystatin C in cell lysates

An ELISA method was used for quantification of secreted cystatin C and cystatin C in homogenates of cells incubated with or without addition of cystatin C to the medium (papers I, II and III). A protease inhibitor cocktail, containing benzamidinium hydrochloride, sodium azide and EDTA, was added to all samples. Triton X-100 (0.2%) was used to prepare cell homogenates. The ELISA results were related to the total protein content of the samples measured by Coomassie Protein Assay.

Purified antibodies from a polyclonal rabbit-anti-(human cystatin C) antiserum was used for capture of the antigen in the original method. By using a primary polyclonal antiserum the probability of binding all variants of cystatin C was increased. For detection, a second monoclonal mouse-anti-(human cystatin C) antibody labelled with biotin was added, followed by horseradish peroxidase conjugated streptavidin (HRP-streptavidin) and a substrate. Recombinant cystatin C ranging from 0.78-100 ng/mL was used for the calibration curve. The exact binding site for the secondary monoclonal antibody is not known, but all cystatin C variants tested so far are recognized, as well as cystatin C in complex with papain (159).

To exclusively detect the internalised cystatin C we used a modified ELISA method (papers II and III). We incubated cells with biotin-labelled cystatin C and could thus detect it in the cell extracts without the biotinylated antibody. HRP-streptavidin could in this alternative ELISA bind directly to the labelled cystatin C before the substrate was added.

The degree of labelling is important when labelled cystatin C is used, either the labels are biotin or fluorescent molecules. Mass spectrometry analysis revealed that wild-type cystatin C was typically labelled with three biotin molecules (range 1-5), which bind to lysine residues. The degree of labelling of the variants of cystatin C can vary due to structural differences, which may expose lysine residues in normally hidden parts of the molecule. To address this problem we used unlabelled cystatin C variants for the uptake studies.

In order to compensate for differences in cell density of the cell cultures, all ELISA results were related to total protein content of the cell extract, as measured by the Coomassie Protein Assay and presented as ng cystatin C/mg protein. Typically the protein concentration in a cell lysate was about 0.5 mg/mL.

Fluorescence

In brief, a fluorophore is a molecule that will absorb light (be excited) when illuminated with light of a defined wavelength. When the electrons fall back to the normal energy state, the absorbed energy is emitted as light of a characteristic lower wavelength. This phenomenon is used in both flow cytometry and fluorescence microscopy. Different
fluorophores can be used at the same time but in these cases it is important that the signals from the fluorophores are totally separated and collected in different channels.

**Flow cytometry**

In flow cytometry a stream of fluid with suspended cells passes a laser light beam and causes scattering of the light. The scattered and emitted light from each cell is measured by various detectors. The fluorescence can emerge from endogenous fluorescent molecules on the cell surface or from fluorescence-labelled molecules added to the cells. The detected forward scatter depends on the cell volume and the side scatter of other cellular properties, such as granulation. The signal from each cell is plotted in a diagram, from which the cells that should be included in the analysis are selected (called gating of the cells) (Fig. 8). By comparing the signal from the treated cells with the signal from untreated cells it is possible to compensate for the cells own fluorescence (autofluorescence).

The degree of labelling of proteins by fluorophores is a key factor when the fluorescence of different proteins is compared. The labelling of a protein depends on exposed lysin residues which may change when, for example, an amino acid substitution is introduced, in analogy with biotin (see above).

Initially, fluorescently labelled cystatin C was used to detect internalised cystatin C by flow cytometry in five cell lines (paper I). Cells were incubated with or without addition of labelled cystatin C to the medium for different periods of time. Trypsin-EDTA was used to detach the cells and simultaneously cleave cystatin C that was attached to the cell surface, but not yet internalised. The gating was set to include about 85% of the cells in the sample (Fig. 8).

![Fig. 8](image-url) Scattergram from a FACS Calibur flow cytometer. The gated cells are included in the analysis. The x-axis shows forward scattering (due to cell volume) and the y-axis shows side scattering (due to granulation, shape and size of nucleus).
**Fluorescence microscopy**

In fluorescence microscopy fluorophores are used to detect certain molecules, organelles or structures in cells. The microscope is equipped with a set of filters which enables the right wavelengths for excitation or emission of the fluorophores to be chosen. The light beam is focused on the specimen through the objective, which also collects the weak emitted light from the fluorophores before the signal reaches the detector, which could be the eye or a camera. It is important, when using multiple fluorophores, that the wavelengths for excitation and emission are separated. This is achieved by the filter pairs used. Images are taken separately for each individual channel and merged with the overlay function of the software.

Epi-fluorescence was initially used to detect internalised fluorescently labelled cystatin C. Cells were incubated with cystatin C, followed by staining with the nuclear markers DAPI, propidium iodide or Sytox® Green Nucleic Acid Stain. By epi-fluorescence the signal from the whole cell is detected in contrast to confocal laser scanning microscopy (CLSM), where resolution and contrast are increased as a consequence of sectioning of the specimen into optical slices. The optical sectioning makes it possible to analyse if the detected fluorescence really is intracellularly derived and the degree of co-localisation with markers for cellular organelles or molecules (paper I) (Fig. 9).

![Fig. 9](image)

**Fig. 9** Uptake of fluorescently labelled cystatin C in MCF-7 cells shown by confocal microscopy. The internalised cystatin C is seen as vesicles (green). Nuclei stained with DAPI (blue).

Cells were incubated with a primary rabbit-anti-(human cystatin C) antiserum and a secondary fluorophore-labelled goat-anti-(rabbit IgG) to detect the cystatin C produced by the cells in a procedure called immunocytochemistry (paper II). Endogenous cystatin C, co-located with cellular organelles, was discovered by additional staining with fluorescent markers of the organelles.
The subcellular localisation of internalised cystatin C was shown by CSLM, as well as co-localisation with intracellular target enzymes (paper III). First cells were incubated with the fluorescence-labelled cystatin C, then the protocol for immunocytochemistry was performed, and finally staining with the nuclear marker DAPI.

Non-labelled cells were used as controls of autofluorescence and the background staining from the antibodies were controlled by omission of the primary antibody or antigen absorption.

**Western blotting**

Western blotting was used to ensure that the internalised cystatin C was intact and recognized by the anti-cystatin C antibody (papers I and II). After incubation with cystatin C the cells were lysed, and the proteins were separated by 4-12% SDS-PAGE. SDS was added to the samples which resulted in negatively charged proteins, that were separated due to molecular size; the smaller the protein, the faster the passage through the gel. To be able to identify the proteins a molecular weight marker was included as well as the recombinant protein. Possible complexes between cystatin C and target enzymes in the cell extracts were dissolved by reducing conditions (achieved by DTT) and incubation of the samples at 96°C for 5 minutes in the denaturing compound SDS.

Subsequently, the separated proteins were electroblotted to a protein-binding membrane. Non-specific labelling of the membrane was blocked and the membrane was incubated with anti-cystatin C antibodies. The secondary antibody was labelled with HRP and visualised by chemiluminescence.

**Enzyme activity assays**

To address the question if the internalised cystatin C still was a functional inhibitor the fluorescent substrates Z-Phe-Arg-NMec, for degradation by cathepsins in general, Z-Arg-Arg-NMec, for specific degradation by cathepsin B, and Z-Ala-Ala-Asn-NMec for specific degradation by legumain were used. Enzyme cleaving of the substrate causes liberation of NMec, which leads to an increase of fluorescence in the sample well, proportional to the degree of activity of the enzymes in the sample.

First we calculated the total inhibitory capacity of the cell lysate. The endogenous cysteine proteases in the cell lysates were denatured and inactivated by incubation at 95°C for 5 minutes. Cystatins are stable proteins and do not lose the inhibitory capacity by this procedure (87). Various volumes of the lysates were then added to a fixed amount papain and the appropriate fluorescent substrate. The fluorescence, derived from cleaving the substrate by the enzyme, was measured and plotted against the volume of lysate used. The volumes of the irreversible inhibitor E64, used as a control, and lysate needed to
abolish enzyme activity was compared and used to calculate the concentration of cysteine protease inhibitor in the samples as E64, like cystatin C, binds to papain in a ratio of 1:1 (paper I). Papain is a non-human model enzyme and to examine the inhibitory capacity of human cysteine proteases cathepsin B was used.

In paper III the cathepsin and legumain activity in cell lysates was measured after uptake of cystatin C administered in different doses (1 and 5 µM), and compared with the activity in control cells (no addition of cystatin C). Cells were cultured in 96-well plates and thoroughly washed before lysis directly in the well. The different substrates were added to the cell lysates and the fluorescence was measured. Enzyme activity was measured as an increase of fluorescence per minute and then correlated to total protein content of the lysate, as measured by Coomassie Protein Assay, reflecting the cell density in the well.

**Invasion and migration in MatrigelTM invasion chambers**

Matrigel™ invasion chambers are made of a thin membrane with 8-µm pores covered by a layer of extracellular matrix component mixture, derived from Engelbreth-Holm-Swarm mouse sarcoma (here called matrigel). The chambers are placed in wells of a culturing plate and starving cells are seeded on top of the matrigel layer. When incubated at 37°C the cells start to invade the matrigel due to a chemoattractant in the bottom well. After incubation the cells that have invaded and migrated through the matrigel are stained and counted, alternatively stained and lysed, before measuring the absorbance of the lysed cells. Cell migration can be studied in a similar way, by the same plastic membranes, but without matrigel.

The invasion and migration properties of MCF-7 cells were studied after incubation for 20 hours with or without cystatin C addition to the medium (paper III). The cells attached to the lower surface of the membrane were stained and lysed and the absorbance was measured. The absorbance of the cells cultured without inhibitor was set to 100%.

**Results and discussion**

**Uptake of cystatins**

*Catstatin C*

The internalisation of cystatin C was demonstrated in different cell lines by various techniques. Initially we started with five epithelial cell lines: MCF-7, MDA-MB-453, MDA-MB-468, A431 and Capan-1 (paper I). Flow cytometry showed an increase of fluorescence in all cell lines after incubation with fluorescently labelled cystatin C. The
cells were grown in adherent cultures and incubated for different time periods ranging from 10 seconds to six hours. The increase of fluorescence continued throughout the whole experiment. The values at 10 seconds represented the efficiency of the washing of unbound fluorophore, and six hours were used because this was the time used by Wassélius et al. (2005) when they detected uptake of cystatin C in cells of a cystatin C-deficient mouse after injection of fluorescently labelled inhibitor (156). The experiment was terminated by addition of trypsin, which detached the cells from the flask and cut off cell surface proteins, including any cystatin C that had not yet been internalised. When the experiment was repeated at 4°C no increase in fluorescence was detected, indicating that the uptake is a specific and active process. ELISA was used to quantify the cystatin C content in extracts from Capan-1 cells incubated with or without cystatin C. This quantitative method also showed that cystatin C was rapidly internalised and reached 4-6 times the basic level after six hours.

Biotinylated cystatin C was introduced, when five neuroblastoma cell lines (LA1-5s, SK-N-BE(2), CHP-212, CHP-234, SH-SY5Y) and the breast cancer cell line MCF-7 was analysed (paper II). The labelled cystatin C that had been taken up by the cells, could easily and exclusively be measured by the modified ELISA. Uptake was verified in the neuroblastoma cell lines and cystatin C was internalised most efficiently in LA1-5s cells (Fig. 10). These cells were also found to contain, secrete and express most cystatin C when cultured in standard medium without cystatin C addition.

The uptake of cystatin C was confirmed in MCF-7 cells as well as in three different prostate carcinoma cell lines, PC3, DU145 and LNCaP (paper III) (Fig. 10). Biotinylated cystatin C was used and the experiment continued for 24 hours. In another experiment the molecular turnover of the internalised biotinylated inhibitor was followed. Biotin-labelled cystatin C could be detected in cell lysates up to 24 hours after uptake.

The uptake of cystatin C seems to be a general phenomenon in cancer cells, but is probably also relevant in normal cells. In a previous study by Wassélius et al. (2005) cystatin C-deficient mice were intraperitoneally injected with human unlabelled cystatin C before blood and tissue samples were collected (156). Cystatin C was measured and the highest values were found in plasma two hours after injection. Then it rapidly declined. Cystatin C was also found in several organs, with the highest concentrations in salivary gland and seminal vesicles six hours after injection. By immunostaining of tissues from another cystatin C knock-out mouse injected with cystatin C it was concluded that cystatin C immunoreactivity was seen in most tissues, generally in cytoplasmic granules. Human fluorescently labelled cystatin C, injected in rat eyes was detected in the cornea, ciliary body and retina especially in the epithelial cells. The pattern resembled that of endogenously produced cystatin C (156).

In another study peritoneal macrophages from the cystatin C-deficient mice were harvested and cultured with interferon-gamma (IFN-γ) for activation. Confocal microscopy, ELISA and Western blotting was used to show that unlabelled cystatin C
added to the cell cultures was taken up by the cells. Because cystatin C-deficient mice were used, it strongly implied that the uptake was not a side-effect due to labelling (K. Frendéus, J. Wassélius, H. Wallin, B. Ehinger and M. Abrahamson, unpublished).

The cystatin C concentrations used for both cell incubations and injections in animals were 1-1.5 µM. These concentrations are in the physiological range; less than the normal concentration in seminal plasma (3.7 µM) and higher than in blood plasma (0.1 µM). When incubating cells for confocal microscopy, 5 µM cystatin C was used in order to get a stronger signal in the microscope. Results from confocal microscopy will be discussed in the section "Subcellular localisation".

Fig. 10 Cystatin C uptake in epithelial and neuroblastoma cells. Cells were incubated with biotinylated cystatin C for 24 hours followed by lysis. The amount of internalised cystatin C was measured in the lysates by the modified ELISA method and related to the total protein content. Cystatin C was taken up in all cell lines tested but in various degrees. Each dot represents the mean of duplicate measurements of a single cell culture, and the bars represent the median of the results from the different experiments (n=6-9).

Other type 2 cystatins

It is possible that some of the other type 2 cystatins also can be internalised, by cancer or normal cells, due to the sequence similarities. Preliminary results from ELISA analyses in our lab show that cystatin D is taken up by MCF-7 cells in a dose-dependant way not
interfering with cystatin C uptake. The simultaneous uptake of both inhibitors resulted in co-localisation in endosomes/lysosomes, which was shown by confocal microscopy with different fluorophore labelling of the two cystatins (Fig 11).

Smith et al. (2012) showed, by Western blotting, uptake of cystatin E/M in human embryonic kidney cells (HEK293) when cultured in conditioned medium from cystatin E/M over-expressing murine carcinoma cells (M4C). Additionally, the internalisation of cystatin E/M resulted in decreased legumain activity in lysates from the HEK293 cells (160).

Cystatin F is expressed in high amounts in immune cells (51,52). It has been shown to be secreted as an inactive dimer, which was internalised and converted to an active monomeric form in the lysosomes after secretion. An internalisation of cystatin F was shown in both cell lines and primary cells and was dependent on glycosylation of the inhibitor and mediated through the mannose-6-phosphate receptor (161). Further it has been shown to be intracellularly retained in promyelocytic U937 cells to a higher degree than cystatin C (56).

![Fig. 11](image) MCF-7 cells simultaneously incubated with cystatin C (red) and cystatin D (green), detected by confocal microscopy. Both cystatins are taken up and co-localise in a vesicular way. Nuclei stained with DAPI (blue). Yellow=merged.
Subcellular localisation

Endogenously produced cystatin C

The endogenously produced intracellular cystatin C was studied in LA1-5s, CHP-212, CHP-234 and SK-N-BE(2) neuroblastoma cells by immunocytochemistry (paper II). Confocal microscopy showed the cystatin C produced by the cells as cytoplasmic vesicles, more dense close to the nucleus. SK-N-BE(2) cells were further used in an attempt to illustrate the intracellular cystatin C localisation in patients with hereditary cystatin C amyloid angiopathy (HCCAA). The patients suffering from HCCAA are heterozygous for a variant of cystatin C with a leucine to glutamine substitution at position 68, which makes the inhibitor more prone to intracellular aggregation (83,85). Cells were transfected with expression plasmids containing the full-length wild-type cDNA, the mutated L68Q-cystatin C cDNA, and a vector without insert as a control. The secretion of cystatin C was increased from the transfected cells, but no intracellular accumulation could be seen in any case, although this was detected in L68Q-cystatin C transfected mouse fibroblasts in another study (94). Confocal microscopy showed that the intracellular L68Q-cystatin C co-localised predominantly with the endoplasmic reticulum, which is in agreement with the default route for a secreted protein. Some co-localisation was also seen in acidic vesicles, which might reflect internalised cystatin C.

An alanine to threonine polymorphism of the penultimate amino acid in the signal sequence of cystatin C has been proposed to be a risk factor for age-related macular degeneration and late-onset Alzheimer’s disease. Expression plasmids were constructed encoding wild-type or A25T-mutant cystatin C, fused with green fluorescent protein (162). It was shown that this cystatin C hybrid was less secreted than wild-type cystatin C and that it was subjected to inappropriate intracellular transport and unexpectedly ended up in association to mitochondria. These results have yet not been confirmed.

Externally administered cystatin C

Fluorescently labelled cystatin C was used to examine the intracellular fate of the internalised inhibitor. In the epidermoid carcinoma A431 cells the internalised cystatin C was visualised in vesicular structures all over the cytoplasm, but not in the nucleus or on the plasma membrane (paper I). In about 10% of the cells the staining was very strong. Normally the microscopy is performed on cells after fixation, meaning the cells are dead. By imaging of live A431 cells after internalisation of fluorescently labelled cystatin C the same granular pattern was seen. Further it was shown that the internalised cystatin C co-localised with staining by LysoTracker®, suggesting that the granules represented acidic vesicles like lysosomes.

The granular staining pattern for internalised cystatin C, localised in vesicular acidic compartments was confirmed in MCF-7 cells (paper I and III). After incubation with labelled cystatin C, the cells were fixed, permeabilized and incubated with bovine serum albumin to block non-specific binding sites, before immunostaining with antibodies
against lysosomal enzymes in order to show co-localisation. Antibodies with affinity for the possible cystatin C target enzymes legumain and cathepsin B were used, as well as an antibody specific for the aspartic enzyme cathepsin D. Images were acquired with filters for different wavelengths and then merged to determine co-localisation. A reliable co-localisation was seen for cystatin C and the enzymes cathepsin D and legumain, indicating endolysosomal localisation of the internalised cystatin C, but no co-localisation was detected for cystatin C and cathepsin B. Control experiments confirmed that legumain and cathepsin D and legumain and cathepsin B, respectively, resided in the same vesicles. The co-localisation between cystatin C and cathepsin D may reflect the degradation of cystatin C by cathepsin D reported by Laurent-Matha et al. (2012) (163).

### Uptake mechanism

**Active and specific uptake**

To investigate if the uptake was an active process the initial uptake experiments were performed by flow cytometry at both 37°C and 4°C (paper I). At 37°C an increase of fluorescence was detected in all cells after incubation with fluorescently labelled cystatin C, reflecting the uptake. None of the five cell lines used showed any intracellular fluorescent labelling when incubated at 4°C together with the fluorescently labelled cystatin C. Further on we performed an uptake competition experiment. Unlabelled cystatin C in high concentrations (10, 20 and 50 µM, respectively) was added to the cells just before addition of fluorescently labelled cystatin C. The cells were then incubated for four hours at 37°C. Flow cytometry showed a decreased cell fluorescence with increased concentration of unlabelled cystatin C. The overall results indicate an active and specific uptake of cystatin C.

**Cystatin C properties important for uptake**

To clarify properties important for cystatin C uptake 10 expression plasmids encoding for variants of cystatin C were constructed (paper III). Because positively charged or hydrophobic amino acid residues were considered important for uptake (164-166) some of these were substituted. Most of the amino acid replacements were located in the enzyme-binding sites. Including wild-type cystatin C and a truncated variant, where the 10 first amino acids in the N-terminal part were cleaved off, we had altogether 12 cystatin C variants in the study.

The uptake experiments were performed in the same way as described previously; MCF-7 cells were incubated with 1 µM of the different inhibitors for six hours. In each experiment the wild-type cystatin C was included and its uptake was set to 100%, and all other results were correlated to this (Fig. 12). The variants with substitutions of lysine or arginine residues in the N-terminal part of the binding site for papain-like cysteine cathepsins (Fig. 6), K5A-, R8G-, and (R8G, L9G, V10G)-cystatin C, were all less internalised than the wild-type cystatin C (median values 12, 1, and 18%, respectively).
The binding site for papain-like cysteine cathepsins includes a tryptophan residue at position 106 in the second hair-pin loop of the cystatin C molecule (Fig. 6). This turned out to have a major impact for uptake because when substituted for a glycine residue the uptake was hardly detectable (variants (R8G, L9G, V10G, W106G)- and W106G-cystatin C) and when substituted for a phenylalanine residue (W106F-cystatin C) the uptake was 168% compared to the wild-type inhibitor. In N39K- and N39A-cystatin C the key amino acid residue for legumain inhibition was replaced (Fig. 6), which indicated the importance of charge for uptake, as N39K-cystatin C was more efficiently taken up than N39A-cystatin C (median values 77 and 22%, respectively). Two variants of cystatin C had substitutions in parts of the molecule not associated with enzyme binding, (R24A, R25A) and K75A-cystatin C. The double mutant (R24A, R25A)-cystatin C which implicates loss of two charged amino acid residues showed internalisation below the detection limit. The variant K75A-cystatin C, on the contrary, showed more efficient internalisation than wild-type cystatin C with the median value 211%, despite the loss of charge. Even though all cystatin C variants used in the uptake studies are still functional inhibitors, the substitution of the lysine at position 75 could possibly lead to a structural change of the molecule which facilitates the interaction between N39 and an assumed receptor.

**Fig. 12** Uptake of cystatin C variants in MCF-7 cells. Cells were incubated for six hours with 1 µM of the different cystatin C variants, before lysis and analysis of the cystatin C content in the lysates by ELISA. The endogenous cystatin C was subtracted from the values before the results were compared to the uptake of wild-type cystatin C, which was set to 100%. Δ1-10=N-terminally truncated cystatin C, 219/214=(R8G, L9G, V10G, W106G)-cystatin C.
Receptor-mediated endocytosis

In order to study co-internalisation of transferrin and cystatin C we incubated MCF-7 cells with fluorescently labelled molecules of both kinds. We could detect parallel uptake and co-localisation in endosome-like organelles. Transferrin is internalised by receptor-mediated endocytosis via the transferrin receptor. Upon transferrin binding to the receptor, the plasma membrane starts to invaginate and clathrin molecules are bound to form a clathrin-coated pit. Several adaptor molecules are recruited and finally a closed clathrin-coated vesicle is formed. When the clathrin-coated vesicle detaches the plasma membrane uncoating of accessory proteins and clathrin triskelions starts. The vesicle is then ready to fuse with others to become an early endosome and further mature with lowering of the pH, and eventually become a lysosome. Once internalised the iron ions bound to transferrin are released and the transferrin is recycled back to the cell surface together with the receptor. This is not the case for cystatin C, which instead follows the endosomal pathway to finally end up in the lysosomes.

Functional consequences of the uptake

Increased cysteine protease inhibiting capacity

Western blotting performed on lysates of Capan-1 cells suggested that the internalised cystatin C was intact as no bands representing degradation products were seen (paper I). To decide if the internalised cystatin C still was a functional inhibitor, the total cysteine protease inhibitory capacity of cell lysates after incubation with or without cystatin C was examined. The concentration of cysteine protease inhibitor in the control samples incubated without cystatin C could be calculated to \( \approx 200 \) pmol/mg protein, mainly due to the major intracellular cysteine protease inhibitor cystatin B (36). In the samples that had been incubated with cystatin C the concentration of cysteine protease inhibitor had increased to \( \approx 250 \) pmol/mg protein, thus the difference represented internalised cystatin C. Additionally, the total inhibitory capacity of cystatin C exposed and non-exposed cells was compared using the model enzyme papain and the human analogue cathepsin B. The enzyme activity was decreased in both cases in lysates of cystatin C exposed cells, reflecting the uptake.

The activities of cathepsins and legumain were found to be dependent on the quantity of internalised cystatin C in MCF-7 cells (paper III). The enzyme activities were lower in lysates of cells that were incubated with a high dose of cystatin C (5 \( \mu \)M) compared with cells that were incubated with a low dose (1 \( \mu \)M) (paper III). Cystatin C is indeed intact and still functional as an inhibitor of both cysteine cathepsins and legumain after uptake. In our study whole cell homogenates have been used. As most of the cysteine proteases are located in lysosome-like compartments, the influence of internalised cystatin C might be even more pronounced in these.
Inhibition of migration and invasion

Matrigel™ Invasion Chambers were used to assess if addition of 1 µM cystatin C to the culturing medium had any effect on migrating and invading properties of MCF-7 cells (paper III). Cells had been starved before seeding in the control or matrigel chambers in order to make them prone to migrate to the bottom well, since this contained medium with addition of 10% fetal calf serum. The cells that had migrated through the membrane were stained and lysed and the absorbance was measured.

Cells incubated with cystatin C or the cell permeable inhibitor E64d were delayed in both migration and invasion compared to cells incubated in standard medium. This was in agreement with other studies showing that incubating MCF-7 cells with either cell permeable or non-permeable inhibitors had an effect on the cells invasive and migrating properties (135,167).

Regulation of intracellular enzymes

The uptake properties of the different cystatin C variants were adapted to study regulation of the intracellular enzyme activity (paper III). Legumain activity in MCF-7 cells incubated with wild-type or W106F-cystatin C was compared to control cells incubated without cystatin C. The legumain activity was significantly decreased after uptake of W106F-cystatin C compared to both wild-type and control cells consistent with the elevated uptake of this variant, 168% compared to the wild-type cystatin C uptake. We were not able to analyse reduced cathepsin activity as the variant W106F-cystatin C is a poor inhibitor of cysteine cathepsins due to the change of the tryptophan residue in the cathepsin-binding region. This could possibly be employed for specific inhibition of legumain, as inhibition of legumain has been shown to inhibit invasion of human melanoma and oral carcinoma cells (50,168). The uptake of W106F-cystatin C is even more pronounced in the prostate cell line PC3, indicating that different cancer cells could be more or less sensitive to this way of regulating enzyme activity.
Overall conclusions

1. Cystatin C is indeed internalised by carcinoma and neuroblastoma cells in a linear fashion up to 24 hours and the turnover of internalised cystatin C is slow.
2. The internalised cystatin C is non-degraded and still biologically functional as a cysteine protease inhibitor.
3. Intracellular cysteine protease activity can be regulated by uptake of cystatin C, as well as migration and invasion of the cells.
4. The uptake is dependent on positively charged and hydrophobic amino acid residues, especially the tryptophan residue in position 106.
5. The cystatin C uptake can be modulated by molecular engineering of the cystatin C molecule.
6. Internalised cystatin C is transported to acidic endolysosomal vesicles, in contrast to the endogenously produced cystatin C, which is located mainly in the endoplasmic reticulum.

Future perspectives

Our studies of cellular cystatin C uptake has just started and a main goal for the future should be to fully understand the mechanism for the internalisation process and the effects of it. In the future it would be interesting to work with:

1. The challenging task to find a potential receptor responsible for the uptake.
2. Studies on cell behaviour after uptake regarding apoptosis, proliferation and differentiation as well as invasion and migration.
3. Characterization of intracellular cystatin C (and other type 2 cystatins) content and expression in tumour tissue, compared to different target enzymes.
4. Regulation of the intracellular enzymes by extracellularly added cystatin C.
5. Uptake of other type 2 cystatins and its consequences.
Ett nytt sätt att se på cystatin C
(populärvetenskaplig sammanfattning)

Bakgrund


Vi har studerat regleringen av vissa enzymer som normalt finns i lysosomerna. Ett sätt för cellen att reglera enzymernas aktivitet är att använda sig av speciella inhibiter, enzymhämmare, som verkar genom att binda till enzymet, just där substratet (det som skulle klyvas) skulle ha suttit och på så sätt hindra klyvningen. Vi har studerat regleringen av de lysosomala enzymerna cathepsin B och legumain. Hämmarna för dessa kallas cystatiner och det finns flera olika cystatiner hos människan. Mest studerad är cystatin C, som finns i alla vävnader och kroppsvätskor, med högst koncentration i ryggmärgsvätska och sädesvätska.

Balansen är ofta störd mellan enzymer och hämmare i och omkring tumörer, så att enzymaktiviteten är förhöjd. Enzymer, som normalt ska transporterats i lysosomerna, kan i en tumörcell omdirigeras till att hamna vid cellmembranet eller till och med utsändras ur cellen. Det finns också exempel på att halten av cystatiner är sänkt. Utsändrade proteaser kan då i högre grad bryta ner det nätverk av proteiner (extracellulärmatrix), som finns utanför cellerna och på detta sätt underlätta för tumörceller att sprida sig till andra delar av kroppen.

Syfte

I mitt doktorandprojekt har jag sökt svar på följande frågor:

1. Tas cystatin C upp av cancerceller?
2. Var i cellen finns det cystatin C som cellen producerar? Och om cystatin C tas upp, vart tar det då vägen?
3. Hur tas cystatin C upp?
4. Hur påverkar upptaget av cystatin C cellerna?
Resultat


Vi använde mikroskopi för att med hjälp av antikroppar mot cystatin C visa att det normala cystatin C som produceras av cellerna finns i det endoplasmatiska nätverket, såsom de proteiner som ska utsöndras brukar göra. Mikroskopi visade också att det cystatin som tagits upp samlokaliseras med målenzymerna i lysosomerna.


Sammanfattningsvis har det visat sig att vi kan reglera upptaget av cystatin C i cancerceller genom att förändra egenskaper hos proteinet. På så vis kan vi reglera den intracellulara enzymaktiviteten. Vi kan också påverka cancercellernas förmåga att sprida sig genom att tillsätta cystatin C. Detta är bara starten på ett nytt sätt att se på cystatin C, som vi fram till nu ansett vara en uteslutande extracellulär enzymhmämare.

I januari 2003 antogs jag som doktorand och under dessa tio år har jag mött många personer som alla på olika sätt har bidragit till min utveckling som forskare. Jag vill varmt tacka er alla!

Speciellt tack till:

Mina handledare

Magnus, du gav mig chansen och trodde på att jag skulle klara av det och lät mig göra resan på mitt sätt, i min takt. Din generösa, positiva attityd och din avslappnade stil smitter av sig på hela avdelningen. Ingen kan som du alltid hitta något som är bra, t.o.m i de försök som har varit riktigt usla. Du är alltid villig att dela med dig både av din enorma kunskap om cystatiner och olika laborationstekniker. Dessutom försöker du lära mig att det är gott om tid… Tack för allt!


Mina kolleger


göra den här resan tillsammans med er! Det har varit otroligt stimulerande att prata med någon som “förstår”.

Bo Holmqvist, tack för alla de timmar du sattit vid mikroskopet med mig. Vilka fina bilder det blev!

Yvonne Bengtsson, tack för att du en gång placerade mig på ”forskningen”. Jag lyckades hålla mig kvar.

**Alla mina vänner**


**Min familj**

Mamma, jag hoppas att du är stolt över mig. Tack för festen!

Fredrik och Sara, Pernilla, Ellen och Albin, jag är så stolt över er. Tack för att ni finns i min närhet.

Micke, tack för att du varje dag gör ditt allra bästa för att göra mig glad och nöjd.

Jag älskar er!

Detta arbete stöddes av Medicinska Fakulteten, Lunds Universitet och det strategiska forskningsområdet BioCARE och av anslag från ALF, Vetenskapsrådet (nr 05196), Cancerfonden, A. Österlunds stiftelse, Magn. Bergvalls stiftelse och Crafoordska stiftelsen.
References


82. Arnason, A. (1935) Apoplexie und ihre Vererbung, Levin & Munksgaard, Copenhagen


53


