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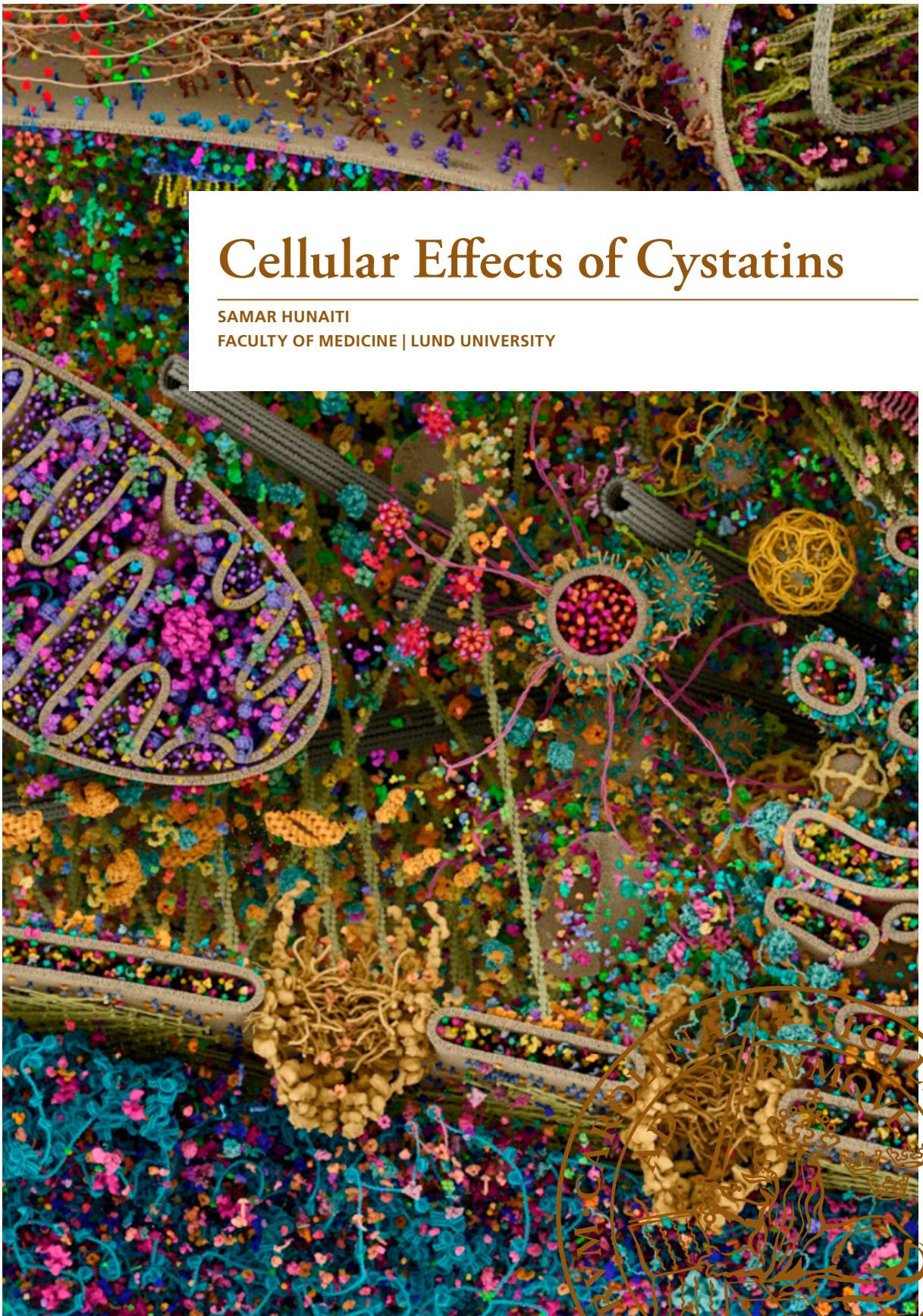

Cellular Effects of Cystatins

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UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

The exact cellular functions of cystatins are still unknown, and the molecular mechanism leading to their internalization into cells is not yet fully understood. Commonly the cystatins are known to inhibit human lysosomal cysteine proteases (cysteine cathepsins) and it is supposed that this is their primary physiological role. The cystatins are traditionally believed to mediate regulation of cysteine cathepsins sometimes leaking out from damaged lysosomes of apoptotic cells and, also, to defend against invading microbial pathogens utilizing cysteine proteases. The balance between cysteine cathepsins and their inhibitors of the cystatin family is apparently pivotal in maintaining a healthy state (cell homeostasis). In several diseases such as atherosclerosis, rheumatoid arthritis, Alzheimer's and Parkinson's diseases and neoplasia this balance is perturbed, with either higher or lower expression levels of the inhibitors or increased or decreased activity of the enzymes. Historically, the concept of cellular internalization of cystatins was indicated from a few studies using chicken egg-white cystatin, which seemed to have intracellular activity when externally added to cell cultures; it inhibited the replication of polio virus. Later, a biologically active human cystatin C was produced in *Escherichia coli*, which was shown to inhibit the replication of herpes simplex- and corona-viruses following its external addition to trans-infected cells. Furthermore, when human recombinant cystatin C was injected intraperitoneally into cystatin C-deficient mice, it was detected within cells in many tissues and organs containing endogenous cystatin C in normal mice.

In this thesis project, we aimed to study the cellular effects of secreted cystatins on cancer cell growth and death, and whether their function to inhibit cysteine cathepsins is associated with these effects. Our studies were focused on the cellular effects of externally added cystatins C and D on viable cell number, cell death, and proliferation under several oxidative stress conditions. A dose-dependent decrease in the viable cell number was observed in leukemic (Jurkat, U937, and HL-60) and epithelial (A375, MCF-7, and PC-3) cancer cells cultured with cystatin C or D. Internalized cystatin D augmented caspase-3-like activity in U937 cells when the intrinsic pathway of the apoptotic cascade was induced by hydrogen peroxide (H_2O_2). In contrast, none of the cystatins showed consistent effects on the extrinsic apoptotic pathway induced by anti-Fas in these cells. Cathepsin C activity was high in U937 cells cultured under normoxia and hypoxia and was significantly inhibited by internalized cystatin D. Induction of lysosomal damage using the cathepsin C substrate, Gly-Phe-β-naphtylamide (GPN), leads to galectin puncta formation, which was used to provide an evidence for the inhibition of cathepsin C by internalized cystatin D within lysosomes. A partial co-localization of cystatin D and galectin was seen using confocal laser scanning microscopy. Inhibition of cathepsin C activity was seen in lysates of U937 cells cultured in the presence of cystatin D or C, which paralleled an observed decrease in cell numbers. This provided complimentary, although circumstantial, evidence that internalized cystatins decrease cell proliferation by a mechanism involving cathepsin C inhibition.

To be able to study the effects of cystatins on the proliferation of individual cells over time, digital holographic microscopy was used for live time-lapse assessment of growth rate and doubling time in A375 melanoma cell cultures. A prolonged doubling time for cells cultured with cystatin C compared to untreated control cells was observed. Tracking of individual cells in holographic images showed that cells incubated with cystatin C underwent fewer mitoses than control cells, which was observable already the first 12 h. The results from incubation of the cells with the two inhibitor variants W106F- and (R24A,R25A)-cystatin C with altered internalization properties supported that internalization is a prerequisite for the cellular effects of cystatins. Preliminary findings showed that externally added cystatin D is as effective as cystatin C in downregulating the growth of A375 melanoma cells.

In all, the results of this thesis demonstrate that 1) Cystatins C and D can reduce the proliferation of cultured cancer cell lines by themselves, in addition to enhancing apoptosis induced by oxidative stress, indicating downregulation of intracellular cysteine proteases; 2) In leukemic cells (U937), internalized cystatin D co-localizes with and targets lysosomal cathepsin C activity, implicating that cathepsin C may be a key enzyme that is involved in the mechanism(s) leading to cystatins' anti-proliferative effects on cancer cells and, possibly also, promoting cell death; 3) Externally added cystatin C and D could prolong the cell cycle and division time of melanoma cells leading to reduced proliferation. These effects on cancer cells were paralleled by the cellular uptake of cystatins, indicating that internalization is a prerequisite for all.

Key words Cystatin C, Cystatin D, Galectin-1, Puncta assay, HoloMonitor, Cancer cells, Apoptosis, Cathepsin C

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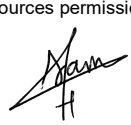
Signature  Date 2021-08-06

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Cover picture: A human cell landscape modeled using nuclear magnetic resonance, X-ray, and cryo-electron microscopy datasets.

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To Dad, Mom, and Dr. Yanal

Table of Contents

Abbreviations

CEW-cystatin	Chicken egg-white cystatin
CLSM	Confocal laser scanning microscopy
CRP	C-reactive protein
DAPI	4',6-diamidino-2-phenylindole
E64	trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane
E64d	(2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutaneethyl ester
ELISA	Enzyme-linked immunosorbent assay
HCCAA	Hereditary cystatin C amyloid angiopathy
HRP	Horseradish peroxidase
LDL	Low-density lipoprotein
LRP	Low-density lipoprotein receptor-related protein
NMec	7-amino-4-methyl-coumarin
RANKL	Receptor-activator of nuclear factor κB ligand
TGF-β	Transforming growth factor β
TGF-βRII	TGF-ß receptor 2
AIF	Apoptosis-inducing factor
DMSO	Dimethyl sulphoxide
Apaf-1	Apoptotic protease activating factor-1
BH	Bcl-2 homologue
CAD	Caspase activated DNAse
CARD	Caspase activation and recruitment domain
DD	Death domain
DED	Death effector domain
Smac	Second mitochondrial activator of caspases
tBid	Truncated Bid
TNFα	Tumor necrosis factor
VDAC	Voltage-dependent anion channel

TRADD	TNF-receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
UVB	Ultraviolet B
DIABLO	Direct IAP-binding protein with a low pI
DISC	Death-inducing signalling complex
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FasL	Fas death receptor ligand
Hsp	Heat shock protein
IAP	Inhibitor of apoptosis proteins
LAMP	Lysosome-associated membrane protein
LAPF	Lysosome-associated apoptosis-inducing protein
LIMP	Lysosomal integral membrane protein
LMP	Lysosomal membrane permeabilization
MMP	Mitochondrial membrane permeabilization
MSDH	O-methyl serine dodecylamide hydrochloride
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PLA2	Phospholipase A2
ROS	Reactive oxygen species
XIAP	X-linked inhibitor of apoptosis proteins

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

Paper I: Hunaiti S, Wallin H, Eriksson M, Järås M, Abrahamson M. Secreted cystatins decrease proliferation and enhance apoptosis of human leukemic cells. **FEBS Open Bio** 2020; 10: 2166-2181. [DOI:10.1002/2211-5463.12958]

In this paper, five different cystatins were investigated, to determine their cellular effects when they were externally added to cultures of three leukemia cell lines. Two of the inhibitors (cystatins C and D) had a substantial effect on growth of the cells and increased caspase-3-like activity following the induction of apoptotic cell death by hydrogen peroxide. The cystatins C and D were also seen to have a distinct effect to reduce cell numbers by inhibition of cellular proliferation.

Paper II: Hunaiti S, Wallin H, Alsafadi HN, Stegmayr J, Danielsson L, Leffler H, Abrahamson M. Inhibition of cathepsin C and lysosomal damage by internalized cystatin D in leukemic U937 cells under oxidative stress. Manuscript 2021

In this paper, we addressed the mechanism of action behind the findings described in Paper I. We chose to continue the studies with leukemic U937 cells and focus on effects by cystatins C and D when the cells were subject to oxidative stress conditions. The activity of various lysosomal enzymes was measured using specific fluorogenic substrates, and it was observed that lysosomal cathepsin C activity was significantly inhibited when cystatins get internalized. It was concluded that this enzyme is likely involved in the anti-proliferative mechanism of cystatins on leukemia cells. Use of galectin puncta assay to study induced lysosomal damage showed that internalized cystatin D can inhibit lysosomal cathepsin C. Confocal microscopy verified a co-localization of cystatin D and cathepsin C within lysosomes.

Paper III: Wallin H, Hunaiti S, Abrahamson M. Externally added cystatin C reduces growth of A375 melanoma cells by increasing cell cycle time. **FEBS Open Bio** 2021; 11:1645-1658. [DOI:10.1002/2211-5463.13162]

In this paper, we shifted the focus from leukemia cells to cells derived from solid tumours (melanoma, breast and prostate cancer), which grow attached to surfaces in culture and hence allow studies by digital holographic microscopy. The studies were focused on effects by cystatin C, as we had generated cystatin C variants with altered internalization properties that should be informative to compare the effects of. The proliferation of the melanoma, breast and prostate cancer cell lines studied was inhibited by cystatin C, in a similar way as demonstrated for leukemic cells in Paper I and Paper II. Detailed studies of A375 cells by digital holographic microscopy demonstrated that internalized cystatin C slow down the cell cycle, leading to delayed population growth for the melanoma cells.

Paper IV: Hunaiti S, Wallin H, Abrahamson M. Effects of cystatin D on growth and death of melanoma cells under oxidative stress. Preliminary manuscript 2021.

In this subproject, we wanted to follow up on results from Paper I and Paper II, demonstrating that cystatin D had the highest inhibitory effect on growth of leukemia cells, by using the technology used for cystatin C studies in Paper III. To this end, we studied the effects of A375 cells by digital holographic microscopy and could conclude that cystatin D is as effective as cystatin C in decreasing melanoma cell proliferation by slowing down the cell cycle, resulting in prolonged cell division time.

Abstract

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Introduction

Cysteine protease inhibitors (**cystatins**) are ubiquitously expressed in mammals, birds, fish, insects, plants, fungi, protozoa, bacteria, and viruses. Cystatins are endogenous, reversible, and competitive inhibitors of the papain-like (clan CA, family C1) proteases, including lysosomal cysteine proteases (**cathepsins**). Some of the cystatins also inhibit asparaginyl endopeptidase or **legumain** (clan CD, family C13) proteases. Maintaining the ratio between cystatins and cathepsins is crucial in the homeostasis of a wide range of physiological processes: antigen presentation, defense against microbial infections, regulation of hormone processing, protein catabolism, bone remodelling, wound healing as well as cell proliferation, motility, survival, and death. Any dysregulation of their expression levels or activities may lead to pathological alterations such as amyloidosis, neurodegeneration, bone resorption, epilepsy, inflammation, host-pathogen reactions, or cancer. Moreover, cystatins may introduce future therapeutic advances in cancer therapy as the levels of some cystatins in multiple tissues and body fluids can, serve as relatively reliable biomarkers (1-16).

Human cystatins

In humans, there are 12 cystatins that belong to family I25 (clan IH) and are classified as such in the MEROPS database (8) based on the presence of the common central conserved amino acid (Gln-Xaa-Val-Xaa-Gly) segment in their sequences, known as 'cystatin motif,' and the presence or absence of disulfide bonds. They are classified as **type 1** cystatins (stefins A and B) if they are non-glycosylated, intracellular, found in the cytosol of different cell types, consist of ≈ 100 amino-acids and lack disulfide bridges. They are classified as **type 2** cystatins (C, D, E/M, F, G, S, SN and SA) if they are predominantly extracellular (i.e., found in various body fluids including plasma, urine, tears, saliva, semen, and cerebrospinal fluid (CSF)), consist of ≈ 120 amino acids and typically contain two intra-chain disulfide bridges in the C-terminal end. They are classified as **type 3** cystatins (L- and H-kininogens) if they are intravascular (i.e., found in blood plasma, synovial and amniotic fluids), large, glycosylated, multi-domain complex (contain three cystatin motifs) with a terminal kinin peptide sequence. Finally, they are classified as **type**

4 cystatins (fetuins A and B) if they consist of non-inhibitory homologs of two type 2-like cystatin domains. (7-8, 17-23).

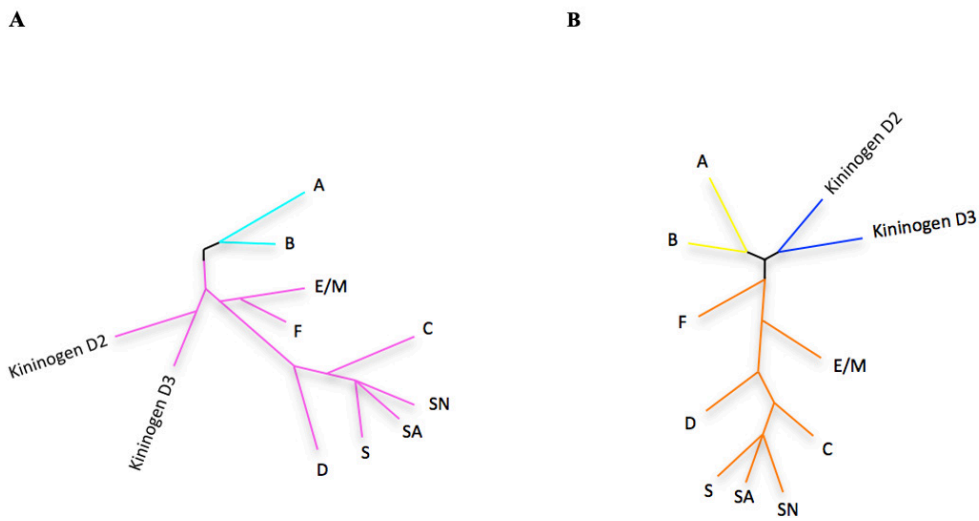


Figure 1 Relationships between human cystatins.
(**A**) Evolutionary tree and (**B**) Similarity tree. Adapted from Abrahamson M. et al. (7).

Human type 2 cystatins

The human type 2 cystatins C, D, E/M, F, G, S, SN, and SA are found in MEROPS subfamily I25B; they are mainly extracellular, secreted proteins and broadly distributed in most biological fluids. Type 2 cystatins are single-chain polypeptides with approximately 120 amino acid residues and are synthesized with 20-26 residue long signal peptides. The mature protein is then transported over the cell membrane into the extracellular milieu. They have two conserved disulfide bridges with a Pro-Trp pair at the end of their C-terminal sequence and there is a distance 10–20 residues between the respective cysteine residues. The 'classic' human type 2 cystatins C, D, S, SA, and SN are more than 50% identical at the amino acid sequence level. In contrast, cystatins E/M, F and G are less than 35% identical at the sequence level (7, 24-32).

The type 2 cystatins, cystatin C and cystatin D

The evolutionary analysis in several studies has shown that cystatin C originated from an ancestor cystatin ~ 650 million years ago (Mya), while cystatin D has evolved from the same ancestor at a more recent origin of ~ 43 (Mya). The cystatin C encoding gene (*CST3*) exists in different mammalian species. On the other hand, the cystatin D encoding gene (*CST5*) is retained only in primates and rodents. Despite the amino acid sequence diversity of cystatins, their genome organization and location in the phylogenetic tree (Figure (1)) suggests a common origin of them all. However, the mechanisms driving their evolution are still unknown but are thought to be for adaptive causes (33-36).

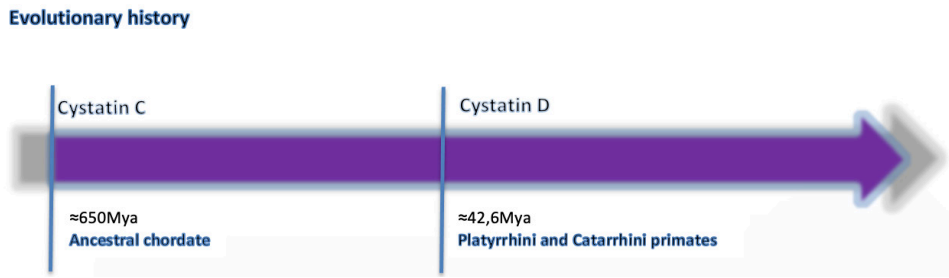


Figure 2 Evolutionarily well-conserved cysteine-protease inhibitors.
Adapted from de Sousa-Pereira P. et al. (36).

Cystatin C

The first cystatin sequence determined was a protein isolated from human urine, called 'γ -trace.' It was later renamed cystatin C, based on similarities to the prototype cysteine protease inhibitor named chicken cystatin, isolated from egg white in 1969. Cystatin C is of small molecular weight, 13-kDa, an essential protein consisting of 120 amino acids encoded by a 7.3-kb gene (*CST3*) located in the cystatin multigene locus on chromosome 20. It is the most abundant type 2 cystatin and is widely distributed in all physiological fluids (predominantly in the seminal plasma) with the highest physiological concentration of ≈ 5 μM (37, 17-20).

In the literature, there is substantial evidence showing cystatin C as a secreted cysteine protease inhibitor, suggested to have roles in regulating bone resorption, neutrophil chemotaxis, tissue inflammation, and resistance to bacterial and viral infections. Cystatin C has been shown to be a sensitive biomarker for early kidney dysfunction, that is not affected by limiting factors such as sex, age, surface area, or muscle mass, in contrast to creatinine that is the conventionally used biomarker for more than 50 years. Moreover, recent studies have shown cystatin C to be a substantial risk marker for death and cardiovascular events in what is called shrunken pore syndrome (SPS) (46). On the other hand, a point mutation in *CST3*,

resulting in the substitution Leu68/Gln (L68Q), causes hereditary cystatin C amyloid angiopathy (HCCAA), a dominantly inherited disorder in Icelandic families. Patients with HCCAA get paralysis and dementia due to successive brain haemorrhages and typically die at 30 years old (27, 38-46).

Cystatin D

Cystatin D was first recognized at the gene level (*CST5*) due to its sequence resemblance with cystatin C. There are two gene alleles coding for it as residue 26 can be either Cys or Arg. Cystatin D has a restricted distribution in the human body; it is highly expressed in the salivary submandibular and paratoid glands. The mature protein comprises 122 amino acid residues and is found in saliva and tears in significant but small amounts. In contrast, it has not been detected in plasma, semen, CSF, or breast milk. Cystatin D was found to be as effective as cystatin C against virus infections. Like cystatin C it probably has a protective role in the host defense mechanisms as they interfere with the process of viral replication (45-51).

Inhibitory functions of cystatins

Cathepsins and legumain

The group of proteases called cathepsins consists of the serine cathepsins A and G, the aspartic cathepsins D and E and the endosomal/lysosomal (family C1) cysteine cathepsins. In humans, there are 11 cysteine cathepsins called cathepsin B, C, F, H, K, L, O, S, V, W and X (or Z). They are known to be unstable at neutral pH, but it is now clear that a few of them remain active at neutral pH for a certain amount of time, varying from minutes (cathepsin L) to hours (cathepsins S). Their active sites consist of a Cys, a His, and an Asn residue. From functional studies on cystatins, their enzyme binding region is partly composed of a segment located near the N-terminal end of their sequences that contains a conserved glycine residue (Gly-11). Side chains of the residues preceding Gly-11, Val-10, Leu-9, and to a smaller extent, Arg-8, are important for optimal cysteine cathepsin binding and of major importance for cystatins' tight-binding properties (7-8, 24, 52-58).

On the other hand, legumain is inhibited effectively by cystatin C but not by cystatin D (59). *In vitro* inhibitory activity studies have shown that the reported inhibitory constant (K_i) values for the individual cathepsin-cystatin interactions are normally varying from 10^{-7} to 10^{-12} M. Cystatin C is the most potent endogenous inhibitor of all cystatins toward multiple family C1 lysosomal cysteine proteases, including cathepsins B, C, H, L and S, when it binds in a 1:1 equimolar inhibitor-protease complex with a competing with a substrate-competitive mechanism. On the

contrary, cystatin D has a narrow inhibitory activity profile, i.e., inhibiting cathepsins C, H, L, and S but not cathepsin B. As these proteases are predominantly present in the lysosomes, while their regulatory inhibitors based on *in vitro* studies are secreted proteins, it is still not evident under which circumstances they can meet if they would (7, 24-32).

The exact cellular functions of cystatins are still unknown and their molecular internalization mechanism into the cells is not yet fully understood. However, numerous studies have demonstrated cystatins as human cancer suppressors while others show that their inactivated genes in cancers such as the cervix, breast, stomach, prostate, and brain will promote cell growth inhibition. For example, in the immune system, cystatins generally exert immunosuppressive effects by downregulating the pro-inflammatory cytokines thus preventing excessive inflammation in wounds. Moreover, cystatin C has a protective role in diseases caused by proteolytic dysfunction such as silicosis, atherosclerosis, or tumor progression (60-62).

However, a report by has been published (63) showing that cathepsin D cleavage of cystatin C favors breast cancer cell progression. Previous studies have shown that cystatin D is located intracellularly close to the nuclear DNA, where it changes the expression of multiple genes that affect various key cellular processes, including migration, adhesion, and signal transduction in human colon cancer cells. It has also been shown that cystatin D overexpression in human colorectal cancer cells has a tumor suppression effect after p53 activation, leading to decreased cell proliferation. Interestingly, cystatin D expression has been reported to be upregulated in human colon carcinoma cells after treatment with vitamin D. Thus, loss of cystatin D is associated with vitamin D receptor downregulation, suggesting its tumor suppressing role (47, 64-66).

Possible roles of cystatins in cancer

Cell healthy state (homeostasis) is controlled by death and proliferation arrest via pivotal pathways removing aberrant malignancy development. As the cell proceeds from one phase to another it is highly regulated by several checkpoints. Defective cells are efficiently taken away by apoptosis from the cell. Therefore, preventing the formation of DNA abnormalities and consequent diseases. perturbation in the balance between cell proliferation and death, may result in cancer. Cancer cells are growing more than needed due to mutations of intracellular proliferation signal pathways. Such mutations enable the cells to re-enter into the cell cycle, independent of positive or negative external stimulus (69-70).

Cancer cell death

To date, there are more than 13 different forms of cell death described, all of which lead to regulated cell death. The loss of lysosomal integrity and mitochondrial membrane permeabilization have been implicated as a decisive step in various forms of cell death. In this thesis project, we will focus on two forms apoptosis and lysosome-mediated cell death (62-63).

Apoptosis

The term (a-po-toe-sis) was first used by Kerr at al. (71) to describe what is now referred to as apoptosis. Apoptosis or "programmed cell death" is a morphologically distinctive, energy-dependent mode of cell death which is responsible for deletion of cells. This happens either normally during growth and aging to maintain cell populations in the body tissues or, pathologically, in response to various stimuli including irradiation, and chemotherapy which cause DNA damage in some cells, leading to apoptosis via a p53-dependent pathway. Apoptosis is carried out by certain cysteine proteases inside the mammalian cell called caspases. There are two main pathways to trigger apoptosis: the intrinsic pathway and the extrinsic pathway (see below for explanation). During apoptosis there are distinguishable morphological features that take place within intact well-preserved cells such as rapid chromatin condensation (pyknosis), shrinkage and budding of the cell, forming membrane-enclosed tightly packed organelles (called, the apoptotic bodies). These are digested by phagocytosis by macrophages or nearby cells. Double-strand cleavage of nuclear DNA at the linker regions between nucleosomes occurs, which is considered as a main biochemical characteristic of apoptosis, leading to the production of oligo-nucleosomal residues (72-73).

Apoptosis is not accompanied by any inflammatory response in contrast to necrosis, which is another morphologically distinct energy-independent mode of cell death that leads to ATP depletion, and cellular swelling (karyolysis) with small protrusions of the cell membrane called blebs. Necrosis is an irreversible breakdown of the cell membrane permeability barrier leading to leakage of metabolic intermediates and cytosolic enzymes. The necrotic cells are the blue non-viable ones seen under the microscope as the blues when adding trypan blue dye in the routine cell count procedure, known as Trypan blue dye exclusion assay for cell counting (74). The two cell death modes overlap simultaneously, independently, and consecutively depending on the nature and/or the degree of stimuli including hypoxia, hyperthermia, radiation, and exposure to cytotoxic agents (75-80).

Caspases

Caspases (Cysteinyl-dependent ASPartate specific proteases) (81-87) are also named (Interleukin 1 β-Converting Enzyme) ICE -like proteases (88-90). To date, caspases (clan CD, family C14) form a family of 14 members so far identified in humans (8). They exist in the cell as inactive proforms or zymogens, which are cleaved to active enzymes, that employ a Cys residue as the catalytic nucleophile. Caspases are specific for cleaving their substrates after Asp residues in target proteins. Their structures were discovered for the first time in 1994, displaying a new protease family (91).

Human caspases are classified as pro-apoptotic caspases which take part in the apoptosis cascade. Depending on their point of entry in the apoptotic pathway, they can be divided into initiators such as (caspase-8, -9 and -10), executioners like (caspase-2, -3, -6 and -7) and effectors which are characterised by the absence of recognisable recruitment domains. Initiators, which are characterised by the presence of CARD (caspase-recruitment domain) or DED (death effector domain) protein-protein interaction motifs at their N-termini, can be further divided into extrinsic (caspase-8 and -10; activated via transmembrane receptors of the TNF (tumour necrosis factor) type-I receptor family once activated they activate effector caspases -3 and -7) and intrinsic (caspase-9; activated by stress, genotoxic damage (91-92). Non-apoptotic caspases, "pro-inflammatory cytokine activation caspases", are stored in the cytosol of cells and are responsible for cytokine maturation by proteolytic activation and release of caspase-1 in response to inflammation or infection. These caspases, for example interleukin-1β (caspase-4) and interleukin-18 (caspase-5), do not take part in the apoptosis pathway (91).

Caspases and apoptosis

Apoptosis, earlier described as a physiologic process of taking away undesired cells, is now seen in diseases like nephrotoxic and ischemic injury, obstructive nephropathy, and polycystic kidney disease. Induction of apoptosis after the disruption of the balance pre-existing in the cell between apoptotic and antiapoptotic proteins like inhibitors of apoptosis proteins (IAPs) can take place via the death receptors. These are cell surface receptors that transfer apoptotic signals commenced by specific ligand binding like Fas ligand, TRAIL and TNFα to its death receptor forming a protein complex (DISC) which results in activation of initiator caspases (caspase-8 or caspase-10) activation within seconds. This is considered the extrinsic pathway of apoptosis as mentioned before.

These initiator caspases can then activate other caspases in the apoptotic cascade, which occurs on the surface of the cell via the death receptors; this pathway is initiated by caspase-8. Side to side, the intrinsic pathway of apoptosis takes place inside the mitochondria via certain chemotaxis primarily cytochrome C and initiated

by caspase-9. This causes the cleavage of vital cellular proteins such as cytoskeletal proteins, eventually leading to activation of effector caspases (caspase-4 and caspase-6) as well. Both pathways converge on the activation of the apoptosis execution level carried out by caspases-3 and -7 (84-85). The two pathways (Figure (3)) together lead to the typical morphological changes in cells undergoing apoptosis mentioned before. Other mechanisms than the activation of death receptors or the mitochondria, by which the caspase cascade is activated in the cells, is via granzyme B, which is delivered to cells by cytotoxic T lymphocytes and is capable of directly activating caspase-3, -7, -8 and -10 (81-85, 91-92).

Caspase-3

Caspase-3 is present in the cell in its inactive form as pro-caspase-3 that is autoproteolytically cleaved by other proteases leading to the assembly of the heterotetrametric enzyme as the active form. Caspase-3 is important for cell death (apoptosis) in a specific, selective manner depending on tissue-cell type, and death stimulus. For instance, some studies (95-96) have shown that caspase-3 knockout mice (which die after few weeks) have skull defects with outer masses of supernumerary cells representing apoptosis failure during development in the brain only, but not in any other tissue, thus demonstrating caspase-3 tissue selectivity. Moreover, it is now well known that caspase-3 is required for common events in death pathways including blebbing, shrinkage, chromatin condensation and DNA fragmentation in all cell types so far examined (97).

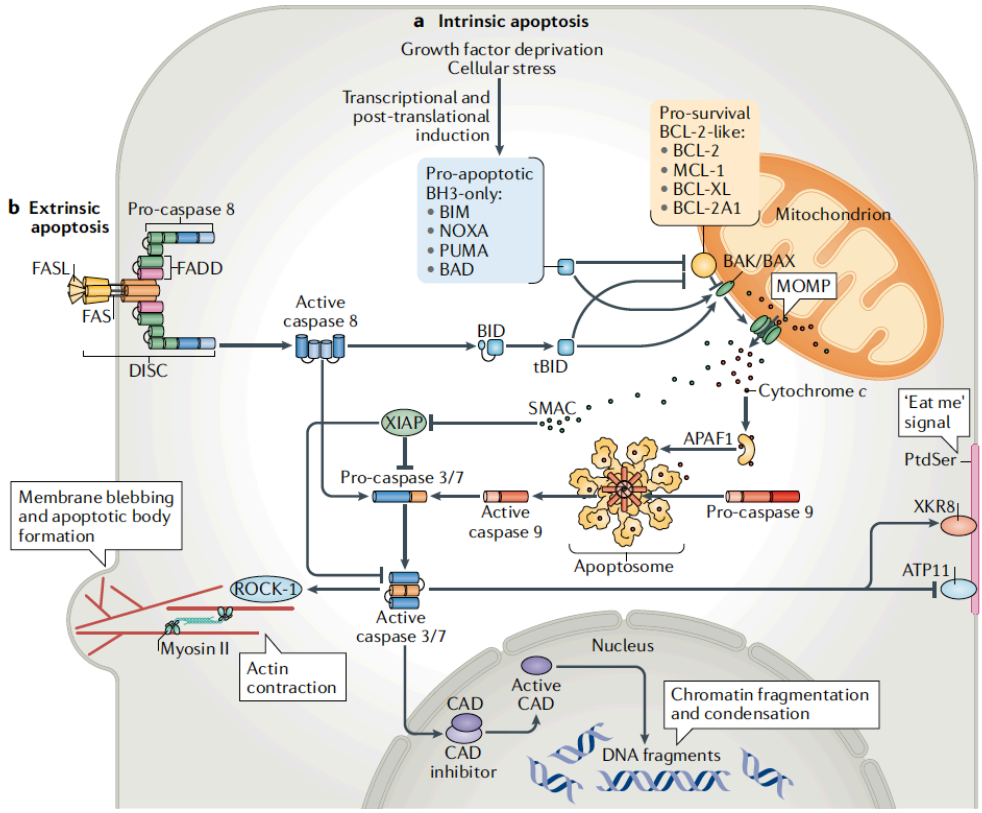


Figure 3 The apoptotic cascade.
Adapted from Bedoui S. et al. (2020) *Nat Rev Mol Cell Biol.* **21**, 678-695.

Lysosomal cell death (LCD)

Lysosomes are acidic cytoplasmic vesicles with low pH (4.5–5), found in hundreds in most cells (98-99). They contain various hydrolases such as lipases, glycosidases, cysteine cathepsins and other proteases. Lysosomes play multiple roles in physiology including responsibility for iron and cholesterol homeostasis, lipid and protein metabolism, endo/exocytosis, plasma membrane repair and cell death. Also, they are involved in pathology including neurodegenerative diseases, inborn lysosomal storage disorders, cardiovascular diseases and cancer. In cancer, cells are more prone to lysosomal destabilization, which possibly is beneficial for the cancer cells to acquire their rapid uncontrolled growth (100-103). Lysosomotropic detergents acting as surfactants, like L-leucyl-L-leucine methyl ester (Leu-Leu-OMe), or reactive oxygen species (ROS), like hydrogen peroxide (H_2O_2), serve as lysosomal membrane permeabilization (LMP) sensitizers, which can cause lysosome-mediated cell death (LCD) downstream (70, 104-106). During oxidative

stress, the mitochondria produce via the respiratory chain is active. When excess of H_2O_2 diffuses into the lysosomes and reacts with redox-active iron, highly reactive intra-lysosomal hydroxyl radicals are generated, which destabilize the lysosomal membrane via the peroxidation of membrane lipids. This results in leakage of their contents including cathepsins from the lysosomal lumen into the cytosol and triggering cell death (70, 107-108). The active sites of lysosomal cysteine cathepsins include a critical Cys residue. They are therefore inhibited irreversibly by the synthetic inhibitor of papain-like proteases E-64d (7-8).

Upon oxidative damage, the intrinsic mitochondrial apoptotic pathway is triggered, resulting in mitochondrial outer membrane permeabilization (MOMP) and LMP-mediated apoptosis. During this, cysteine cathepsins can activate the degradation of the extracellular matrix and various cytokines, growth factors and death chemotaxis such as cytochrome C and the proapoptotic Bcl-2 family members (including Bax, Bim, Bak and Bid) or inhibit the anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-XL and Mcl-1). In Bax/Bak double-deficient mice (106), cell death was absent after LMP (6-12). Yet, the role of cathepsins in promoting LCD depends on cell type, apoptotic-signalling pathway, and variety of stimuli such as death receptors ligation, p53, UV irradiation, and oxidative stress (111). For instance, cathepsin D is associated with cell death in various tissues such as the brain, intestine and retina (112-114). By contrast, knockout mice lacking cathepsins B, C, L or S were viable and fertile with no obvious developmental defects (115).

LCD is mediated by the abundant, highly glycosylated integral transmembrane proteins of the inner lysosomal membrane called lysosome-associated membrane proteins (LAMP) -1 and -2, and by lysosomal integral membrane protein (LIMP) -2. These proteins are responsible for maintaining the integrity of the lysosomal membrane and the acidic pH of the lumen, as well as importing/exporting the generated metabolites wherever needed. The deficiency of these membrane proteins is associated with Danon's disease development, which is associated with the accumulation of autophagic material in the myocytes, resulting in cardiomyopathy and some mental retardation. Moreover, it was found that LAMP-1 and -2 sensitize the lysosomal membrane to apoptotic inducers. Their protein levels are decreased in various cancer types such as breast, bone, prostate, and colon cancer (13-16).

LAMPs are degraded in a cathepsin-dependent fashion, causing increased lysosomal instability and LMP, leading to cytosolic acidification. The pH-dependent proteolytic activity of accidentally released cathepsins is suppressed by cystatins so that, they cannot cause any cellular damage (99, 119-120). LCD is highly conserved in the evolution from yeast to mammals, but its underlying mechanisms remain poorly understood primarily due to the lack of proper detection methods. Currently, the most sensitive method for the detection of LMP is galectin-based method, known as "lysosomal galectin puncta assay" (122-123). Human galectins are a family of 10 soluble β-galactoside-binding lectins with one or two conserved recognition domains. They are found in the nucleus and cytosol of cells

as well as in the extracellular space. The binding of extracellular galectins to cell surface glycans can mediate cell-matrix and cell-cell transmembrane signalling interactions (124-125).

Cancer cell growth

The cell fate is controlled at the cell cycle checkpoints, where the growth-regulating stimuli are interpreted, deciding whether the cell cycle should proceed or not. Dividing cells must move from G1 phase (the first gap) of the cell cycle, into the S phase (DNA synthesis), followed by the G2 phase, then to M phase (duplication of cells, mitosis). The cell cycle checkpoints regulate a complex matrix of interactions, which later determine cell growth, arrest, or death. In cancer, there are fundamental mutations in the genes regulating cell cycle and division, resulting in an uncontrolled cell growth (126-128).

Cystatins in cancer cell death and growth

Cancer is manifested by changes in mRNA and protein expression levels of cystatins, leading to proteolytic dysregulation and cancer growth progression. Cystatin C, is the most investigated cystatin in many *in vitro* and *in vivo* studies. It has been implicated as both cancer suppressor and promoter. For example, recombinant cystatin C decreased cell growth and invasion *in vitro* in human colon carcinoma cells, mainly by cathepsin L inhibition (129). Also, cystatin C has been reported to inhibit tumour growth, metastasis and invasion via cathepsin B inhibition in various cancer types (ovarian cancer, oesophageal carcinoma, human glioblastoma and fibrosarcoma lung cells) (96, 130-132). In contrast to its cysteine cathepsin inhibitory effects in cancer growth, some reports show that it can promote cancer growth and progression via proteolysis-independent mechanisms (133-136). However, some studies showed that cystatin C overexpression caused increased apoptosis in human melanoma cells and fibrosarcoma cells (137) whereas cystatin C deficiency decreased apoptosis by promoting epidermal dysplasia in K14-HPV16 transgenic mice (138). Cystatin C has also been seen to decrease p53-induced apoptosis by reducing cathepsin L activity in HTC-116 human colorectal adenocarcinoma cells (139).

Altogether, several of the studies cited earlier indicate that cystatin functions during cancer cell growth and death is likely not limited to their cysteine cathepsins inhibitory activity. Cystatins may have a complex role as mediators of different signalling pathways.

The Present Investigation

Justification for the present study

The cell journey from birth to death is highly regulated by mechanisms involving different proteins. Defects in the regulation of cell death (apoptosis) contribute to a wide variety of diseases, such as cancer, neurodegeneration and autoimmunity. Increased knowledge about the molecular details of the apoptosis process could lead to novel, more effective, treatments with less serious side effects for these illnesses. The balance between proteases and their inhibitors seems to be crucial to maintain the healthy state of a cell. In many diseases, though, this balance is disturbed, by either increased or decreased expression or activity of enzymes or inhibitors. The protease inhibitors this thesis mainly deals with, cystatins C and D, were until recently considered as exclusively secreted cysteine protease inhibitors, which only exert their effects extracellularly. Recent findings demonstrating that they can get internalized into cancer cells justified a project to elucidate whether cystatins are proteins affecting the mechanisms regulating cellular growth and death.

Aims

The specific aims of this thesis project were:

I. To clarify the possible role of cysteine protease inhibitors (cystatins) and their theoretical target enzymes (lysosomal cysteine cathepsins) are in cell growth and apoptosis.
II. To investigate if externally added cystatins can affect the lysosomal death pathway, which is a promising target for cancer therapeutic intervention.
III. To check whether other secreted cystatins than cystatin C, are internalized into cancer cells and affect their properties.
IV. To find out which of the cysteine cathepsins is(are) the target enzyme(s) for cystatins C and D when these cystatins get internalized and affect cellular growth and death.

Background

When this project started, several lines of work had provided some evidence that externally added cystatins could affect cell proliferation, migration, and invasion of cancer cells, and indicated that this could be due to their internalization into the cells. As to indications for internalization of the cystatins, it had also been shown that when the type 2 cystatins C and D were externally added to cell cultures they inhibited the replication of polio-, herpes simplex- and corona-viruses (137, 57, 50-51), which should be intracellular processes. It had further been shown that when cystatin C was intraperitoneally injected into cystatin C deficient rats, it was internalized into various cell types in the cornea, retina, and ciliary body which normally contain endogenous cystatin C (138). Moreover, it was reported by Wallin and co-workers (2-3, 29-30) that recombinant cystatin C was taken up into different human cell lines such as breast, melanoma and prostate cancer cells. The protein could there be visualized in intracellular endosomal-like vesicular compartments by fluorescence microscopy.

Some studies in the literature had shown that cystatins may be involved in and possibly regulate cell death processes. Cystatin C can induce neuronal cell death *in vivo* and in cultured human CNS neurons (139). Others have reported that cystatin C has a protective effect on high oxygen-induced cell death in cystatin C-expressing PC12 cells (140). Ultraviolet B irradiation (UVB)-induced procaspase-3 cleavage and caspase-3 activation, which are signs of cell death, were suppressed in cystatin A expressing human keratinocytes (141). Moreover, it has been shown that knockdown of cystatin C in different human cancer cells reduced Adriamycin-induced caspase-3 activation (136). These findings all indicated that cystatins could regulate cell death processes but were conflicting with respect to if this regulation resulted in increased or decreased cell death.

Methods

The research carried out in this thesis project is cell-based. All methods utilized are described in detail in the papers attached at the end of the thesis.

Results and discussion

1. Internalization of type 2 cystatins C and D

The main goals of this thesis included to evaluate the cellular effects of secreted cystatins after their external addition to cell cultures, and to independently verifying their localization with the coordinates of their theoretical target enzymes if they ever meet. Of special interest for the present thesis work, the cellular internalization of cystatins into endo-lysosomal like vesicles which has been previously observed in several solid tumour types using confocal laser scanning microscopy (CLSM), immunoblotting and ELISA methods (2-3, 29-30).

In the first part of the thesis project, blood cell-derived cancer cell lines were investigated (Paper I, Paper II). From the concept of cystatin internalization, this provided an opportunity to investigate whether the internalization after the external addition of cystatins to cell cultures is a universal/general phenomenon regardless of cell type. When the internalization of cystatins C and D was studied by CLSM in U937 leukemia cells, a significant uptake and co-localization of both cystatins were visualized as cytoplasmic endo-lysosomal-like vesicles in all cells (Figure 1). It resembled the co-localization of internalized cystatin C and the known endo-lysosomal enzyme, cathepsin D in MCF-7 breast adenocarcinoma cancer cells (30) and, also, the co-localization of internalized cystatin E/M and cystatin C in A375 melanoma cells (2). Follow-up experiments using ELISA were performed to quantify and verify the intracellular content of cystatins C and D in U937 cell extracts. The results showed that both cystatins are internalized in significant quantities (up to 100 ng/mg of cell protein) after 5 h of incubation (Paper I). It has also previously been shown by FACS (3) that cystatin C can be rapidly internalized in different solid epithelial tumour types including A-431, MCF-7, MDA-MB-453, MDA-MB-468 and Capan-1 cells already after 5 min. Taken altogether this demonstrate that the internalization of exogenous cystatins is general for cancer cell lines and that cystatins are internalized in a similar fashion regardless of cancer cell type.

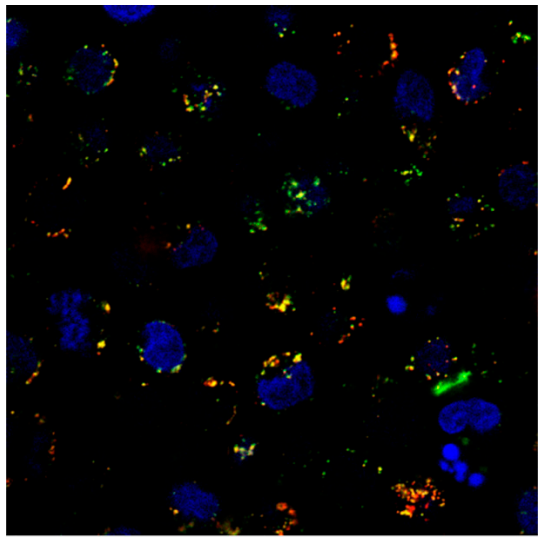


Figure 1 Intracellular co-localization of internalized cystatins C and D in leukemic U937 cells (Paper I).
The cells were simultaneously incubated with 3 μM of fluorescently labelled Alexa Fluor 568-cystatin C (red) and Alexa Fluor 488-cystatin D (green) for 5 h. The internalised cystatins C is seen as red vesicles, the internalised cystatins D is seen as green vesicles and yellow correspond to vesicles wehere both cystatins co-localize. Nuclei were stained with DAPI (blue) using CLSM.

2. Interplay between cystatins and cysteine proteases in cellular processes

2.1. Cystatins and cell death

Some studies in the literature have shown that cystatins may be involved in and possibly regulate cell death processes. For instance, cystatin C induced neuronal cell death *in vivo* and in human CNS neurons cultures (140). Others have reported that cystatin C has a protective effect instead on high oxygen-induced cell death in cystatin C-expressing PC12 cells (141). Ultraviolet B irradiation (UVB)-induced procaspase-3 cleavage and caspase-3 activation, which are key signs of cell death in both the intrinsic and extrinsic apoptotic pathways, were suppressed in cystatin A expressing human keratinocytes (142). Moreover, it has been shown that knockdown of cystatin C in various human cancer cells reduced Adriamycin-induced caspase-3 activation (136). Apparently, these effects of cystatins on cell death are dependent upon oxidative stress induction inside cancer cells. A few previous studies (check Introduction) have reported the ability of some cystatins to

induce apoptosis in several cell types. However, the mechanism by which the cystatins then studied modulate cell death has not been elucidated in detail yet.

*2.1.1. Effects of cystatins on caspase-3 activity in leukemic cell lysates (**Paper I**)*

ELISA performed on U937 cell lysates suggested that cystatins C and D were internalized in significant amounts (Paper I). To decide if the internalized cystatins C and D, and initially also some other cysteine protease inhibitors, can affect active proteases in cell death, the total caspase-3-like activity was measured in cell homogenates after incubation with or without cystatins in the cell culture for 15 h. The proteins studied were the intracellular inhibitor cystatin A, the secreted type 2 cystatins C, D and E/M and chagasin, a protein with structural resemblance but properties like cystatins in *Trypanosoma cruzi* (143). The concentration of cystatins used was 1 μM, which is within the physiological range of cystatin C concentrations (0.1–3.7 μM) in different body fluids. To initiate the extrinsic apoptotic pathway, we simultaneously added 0.2 μg/mL of a monoclonal anti-Fas antibody. Ongoing apoptosis was assessed by measurement of caspase-3-like activity in cell lysates by the fluorogenic substrate Z-DEVD-NHMec, but no significant effects of any of the cystatins were obvious (Figure 2A). The degree of caspase-3 activation in U937 and the other two leukemia cell lines studied in parallel after anti-Fas induction was relatively low. This could be due to that the expression of the Fas receptor (CD95) shown by qRT-PCR analysis of mRNA levels were low (Paper I and data in The Human Protein Atlas (https://www.proteinatlas.org/)).

This led us to induce the other classical apoptotic pathway via mitochondria by adding H_2O_2 at a concentration of 40 μM - which is known to induce in U937 cells (144-146). The cystatins were simultaneously added at a concentration of 1 μM in a similar fashion as for anti-Fas induction. Interestingly, the caspase-3-like activity augmented by all cystatins under oxidative stress conditions (Figure 2B), suggesting an increased in apoptosis via the mitochondrial intrinsic pathway in U937 cells. Chagasin showed no consistent effect on caspase-3-like activity when induced by any of the two the apoptotic pathways (Figure 2).

To verify that the effect of cystatins is limited to viable cells we carried out a control experiment, where we used homogenates from cells in which apoptosis had been induced for 15 h prior to the addition of cystatins, i.e., they were added to the caspase-3 assay just before substrate addition. No increase in fluorescence by any of the cystatins studied was observed in comparison to untreated control cells, suggesting that cystatins cannot affect caspase-3 activity directly. Rather, they seem to enhance an already induced apoptosis, and viable cells is a prerequisite for the boosted apoptosis (Paper I).

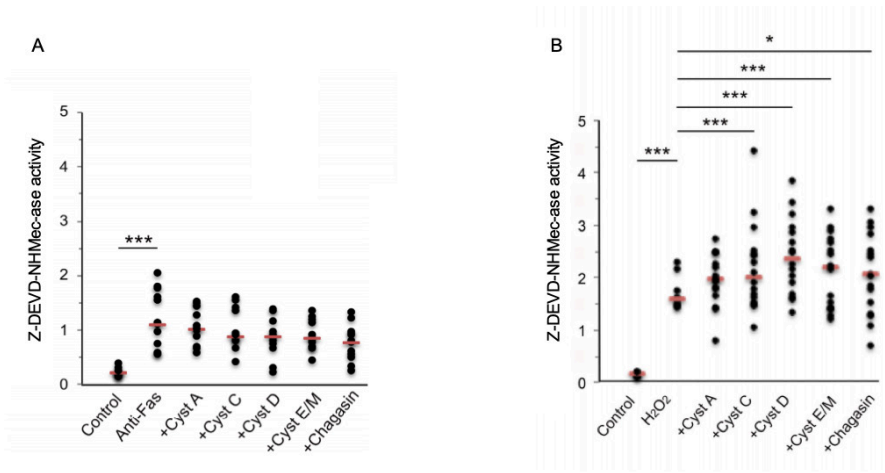


Figure 2 Screening of different cystatins' effects on caspase-3 like activity in leukemic U937 cells (Paper I). **(A)** The extrinsic apoptotic pathway was induced via 0.2 µg/mL anti-Fas antibody. **(B)** The intrinsic apoptotic pathway was induced by 40µM H_2O_2. The cells were incubated for 15 h with anti-Fas and or H_2O_2 as well as 1 µM of cystatins A, C, D, E/M, or chagasin. Raw assay data from 3 to 4 independent cell experiments were grouped, with red bars indicating median values. Groups of data were compared and analyzed statistically by Mann–Whitney test. Significant differences (*P < 0.05; ***P < 0.001)

To seek independent evidence for a stimulation of intrinsic pathway apoptosis by externally added cystatins we analyzed annexin V, known to be cell death marker. The inhibitors selected were cystatin D, as its effect on the intrinsic apoptotic pathway was the most pronounced, and cystatin C, which also showed a consistent effect on apoptosis as well as being the most highly expressed cystatin in the U937 cells. A significant increase in the cell surface phosphatidyl serine as is a marker for apoptosis was measured by flow cytometry using fluorescently labeled annexin V and simultaneous addition of 1 or 5 µM cystatin C (Figure 3A) or cystatin D (Figure 3B) with 40 µM H_2O_2.

Incubation with only H_2O_2 increased the amount of annexin V-positive cells (Figure 3). When the cystatins were externally added to U937 cell cultures they further increased the amount of annexin V-positive cells and their effects on apoptosis were more evident at a higher concentration, especially in the case of cystatin D (Figure 3, B). Addition of the cystatins only at the same concentrations without H_2O_2 apoptosis induction of the U937 cells resulted in no significant effects on the amount of annexin V-positive cells (data not shown).

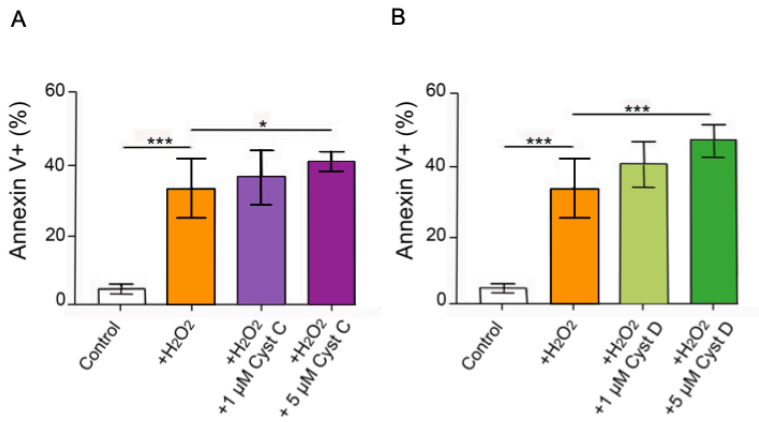


Figure 3 Augmented apoptosis in leukemic U937 cells by cystatins C and D by annexin V staining (Paper I). The cells were incubated for 24 h with 40 µM H_2O_2 and 1 or 5 µM cystatin C **(A)** or cystatin D **(B)**. Apoptosis was determined by flow cytometry using annexin V-APC staining. Flow cytometry measurements were made in triplicate wells in three independent experiments. The bars show mean results, with error bars representing SD. Statistics were calculated by Mann– Whitney test (*P < 0.05; ***P < 0.001) (Modified figure with data from Paper I.)

2.2 Cathepsins

Some cellular proteases are believed to play relevant roles in mediating various apoptotic responses. It has been previously proposed that cystatins are playing the good guy role in having a protective effect effective in preventing TNF-induced cell death in a monocytic cell line (147-148). However, in a recent study on B16F10 melanoma cells cystatin C promoted cell growth and metastasis by proteolysis-independent mechanisms (149). Maybe this could be explained by that the loss of one cathepsin is compensated by another, supported by a study (150) showing that both E-64 and cystatin C increased levels of active cathepsin S whereas they inhibited active cathepsin L in breast carcinoma cells as a compensatory mechanism. Thus, cysteine cathepsins seem to have a complex and dual role in cancer affecting both cell growth and death.

2.2.1 Effects of oxidative stress on cathepsin expression levels ***(Paper II)***

We investigated a possible effect of hypoxia on the expression levels of the 11 human lysosomal cysteine cathepsins, in the U937 leukemia cells. Legumain was also investigated, as cystatin C has a second enzyme binding site with affinity for this enzyme (7, 59). Our initial analysis using qRT-PCR assay showed that in

normoxia, cathepsin C was expressed at the highest mRNA levels and that cathepsins B, H, S and Z were also expressed, in good agreement to data in The Human Protein Atlas (https://www.proteinatlas.org/). The mRNA levels of cathepsins F, K, L, O, V, W and legumain were below detection limits. This implicated that the later cathepsins (and legumain) are unlikely to be the target enzymes for inhibition by cystatins after their internalization. The qRT-PCR data showed that hypoxia caused minor differences in mRNA expression levels of some cathepsins, with the highest relative up-regulation of cathepsins C and H expression (by ~50%) (Figure 4).

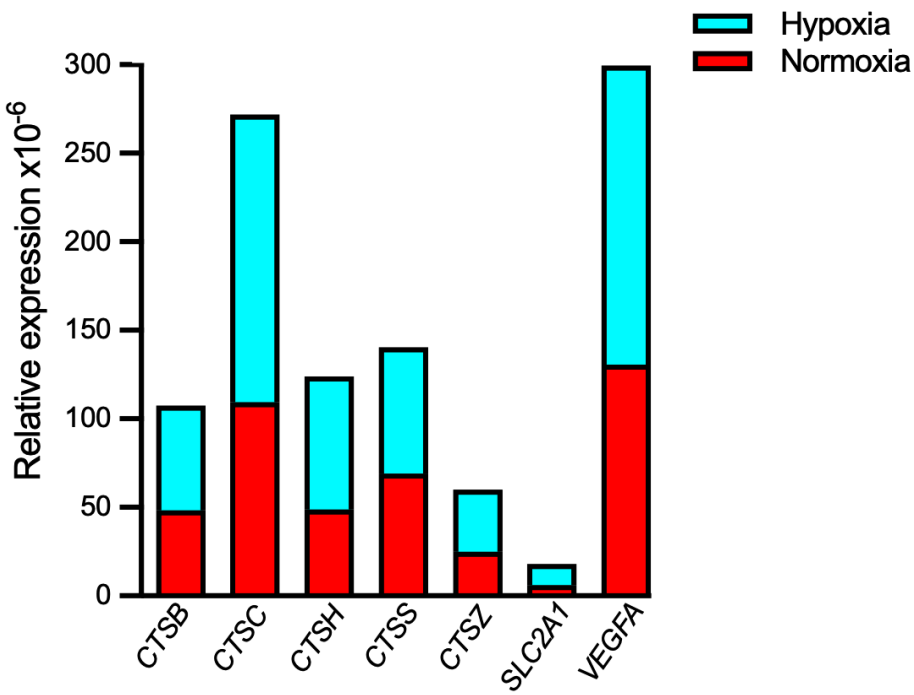


Figure 4 Screening of cathepsins gene expression in leukemic U937 cells by mRNA quantitation (Paper II).
The cells were cultured in normoxia or hypoxia for 24 h. The relevant mRNA levels were measured by qRT-PCR in relation to 18S rRNA. The probes used were for *CTSB* (cathepsin B), *CTSC* (cathepsin C), *CTSH* (cathepsin H), *CTSS* (cathepsin S), *CTSZ* (cathepsin Z), *SLC2A1* (glucose transporter-1; GLUT-1) and *VEGFA* (vascular endothelial growth factor-A). Data from two experiments (with 1-3 wells in each) are shown as Bars represent the mean of the mean values from the four experiments under normoxia compared to hypoxia.

2.2.2 Intracellular activity of cathepsins C and H under oxidative stress ***(Paper II)***

To investigate the role of cysteine cathepsins in H_2O_2-induced cell death in U937 cell cultures, we analyzed their proteolytic activity in cell lysates after the external addition of cystatins C and D to the cell cultures for 15h. In earlier studies, it was taken as evidence for internalization that cystatin C and its variants with altered uptake/proteolytic inhibition characteristics were shown to inhibit intracellular cathepsin B and legumain activities associated with cancer cell invasion and metastasis in MCF-7 PC3, DU145, LNCaP, MDA-MB-435S, A375, and C8161 melanoma cells (2, 30).

First, we analyzed cathepsin C-like activity using the dipeptidyl peptidase substrate H-Gly-Phe-NHMec (Figure 5A) in U937 cell cultures under normoxia. This demonstrated a relatively high activity (median value of 5.83 FU/min/mg protein), in accordance with the relatively high *CTSC* gene expression level in these cells. The median values were clearly decreased in cell homogenates incubated earlier with externally added cystatins C and D (to 3.56 and 1.08 FU/min/mg protein, respectively), compared to control. Oxidative stress induced by H_2O_2 caused a further reduction of enzyme activity by cystatins C and D addition (Figure 5A). While under double oxidative stress conditions (hypoxia and H_2O_2 addition) the cathepsin C-like activity was also decreased compared to control ($p<0.001$) and was further reduced in cells incubated with cystatin D but not when cells were incubated with cystatin C. But no additional decrease by hypoxia in the total enzyme activity was seen and in the presence of cystatin C (Figure 5B).

We then analyzed the activity of the second highly expressed cysteine cathepsin in U937 cells under normoxia which is the exopeptidase cathepsin H, using its specific substrate H-Arg-NHMec. The cathepsin H-like activity was relatively low with a median value of (0.68 FU/min/mg protein). There was no inhibition of activity caused by external addition of cystatin C or cystatin D addition to U937 cells, neither when oxidative stress was induced by H_2O_2 nor in control cells (Figure 5C).

Although the measurements of cathepsin H-like activity under normoxia showed no effect of cystatins C and D. We wanted to investigate cathepsin H-like activity under hypoxia, as the ratio of cathepsin H mRNA expression under hypoxia compared to normoxia was increased (Figure 4). This was reflected by a two-fold activity increase in control cells under hypoxia compared to normoxia. On the other hand, when cells were incubated with cystatins C or D no inhibition of cathepsin H-like activity was observed (cystatin C rather increased the activity slightly), not even when they were further stressed by H_2O_2 induction (Figure 5D).

In all, there was an effect on some intracellular cysteine cathepsin activities by cystatin C and especially by cystatin D when they were externally added to cell cultures. This was most pronounced for cathepsin C activity in both normal and

H_2O_2 stressed U937 cells. In cells stressed by hypoxia, however, the cystatin effects were less pronounced, U937 cells showed a low sensitivity towards the hypoxic condition than solid tumors (151). As differences in the inhibition of intracellular proteolytic activity by cystatins in normoxia compared to hypoxia is almost the same. Thus, in the following experiments we continued with normoxia conditions only.

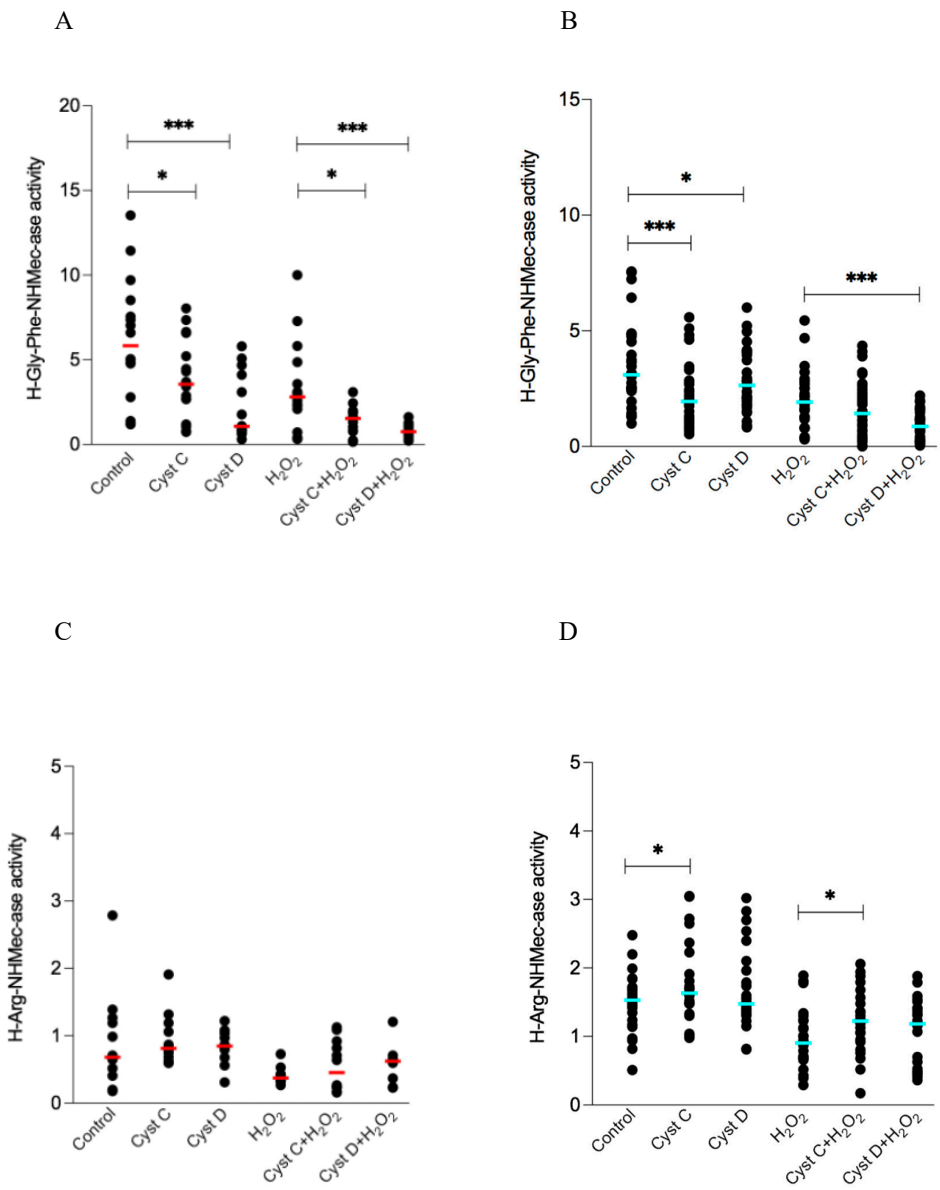


Figure 5 Inhibition of cysteine protease activity in leukemic U937 cells under normoxia and hypoxia (Paper II). The cells were incubated with medium containing 1 μM of cystatins C or D for 15 h, with and without simultaneous H_2O_2 induced oxidative stress under normoxic or hypoxic conditions. The activity of cysteine proteases was measured in lysates of the cells, using (A) the cathepsin C-specific substrate, H-Gly-Phe-NHMec under normoxia; (B) the cathepsin C-specific substrate, H-Gly-Phe-NHMec under hypoxia; (C) the cathepsin H-specific substrate, H-Arg-NHMec under normoxia; and (D) the cathepsin H-specific substrate, H-Arg-NHMec under hypoxia. Activity was expressed as FU/min/mg cellular protein. Raw assay data from four independent experiments were grouped. *Horizontal red* and *baby blue lines* indicate median values under normoxia and hypoxia respectively. Groups of data were compared by Mann-Whitney test and the significance is indicated in the figure (*p<0.05 and ***, p<0.001).

2.2.3. Cathepsin C in LCD

From the proteolytic activity experiments shown in (Figure 5), we could conclude that cathepsin C appeared to be a candidate as the primary target enzyme for cystatins internalized into leukemic cells. We could also conclude that internalized cystatin D had the most distinct effect on its activity, whether the cells were stressed or not. We had earlier in (Paper I) demonstrated that caspase-3-like activity is augmented in U937 leukemia cells in the presence of cystatin D, when cellular stress leading to apoptosis was induced by H_2O_2 (Figure 2).

A recent study (152) demonstrated a rapid cathepsin C-dependent LMP in U937 cells triggered by Leu-Leu-OMe, a well-known lysosomotropic agent, which was followed by the release of other cysteine cathepsins into the cytosol leading to apoptosis. We chose to use another, well-established way to chemically induce lysosomal damage using GPN, which a synthetic substrate of cathepsin C that accumulates inside the lysosomes, where it is specifically hydrolyzed by the lysosomal cathepsin C to charged amino acids that are trapped in the lysosomes, causing osmotic pressure. The phenomenon of rapid accumulation of galectins - particularly galectin-3 - around disrupted and damaged intracellular vesicles (in response to challenge by bacteria or chemical agents) and the formation of galectin puncta, was first discovered in phagosomes disrupted by the cytosolic pathogen *Shigella* (153).

GPN at a concentration of 0.3 mM has in later studies been shown to induce galectin-1, -3, -8 and -9 accumulation around damaged lysosomes in HeLa cells, following treatment for 10 min at RT (154-158). It has been shown that myeloid cells express more mRNA for galectin-1 than for galectin-9 while none of the other lectins belonging to this family are expressed (159). Thus, we have here analyzed galectin-1 puncta to assess LCD under oxidative stress, as its expression is higher in the U937 cells than for galectin-3 (www.proteinatlas.org).

First, U937 cells were cultured with or without H_2O_2, and GPN, then immunostained to detect galectin-1 accumulation as puncta by CLSM. In untreated control cells cultured in standard medium, almost no galectin-1 puncta could be detected. GPN did induce lysosomal damage in the cells as evidenced by visible galectin-1 puncta. When cells were treated with H_2O_2, without the external inducer GPN, the puncta

assay gave evidence for lysosomal damage, in good agreement with earlier studies on oxidative stress and lysosomal damage (119-120, 160-16). Moreover, we investigated the induction of lysosomal damage by the combination of H_2O_2 and GPN. There were no indications for an additive effect by the two inducers (Figure 6).

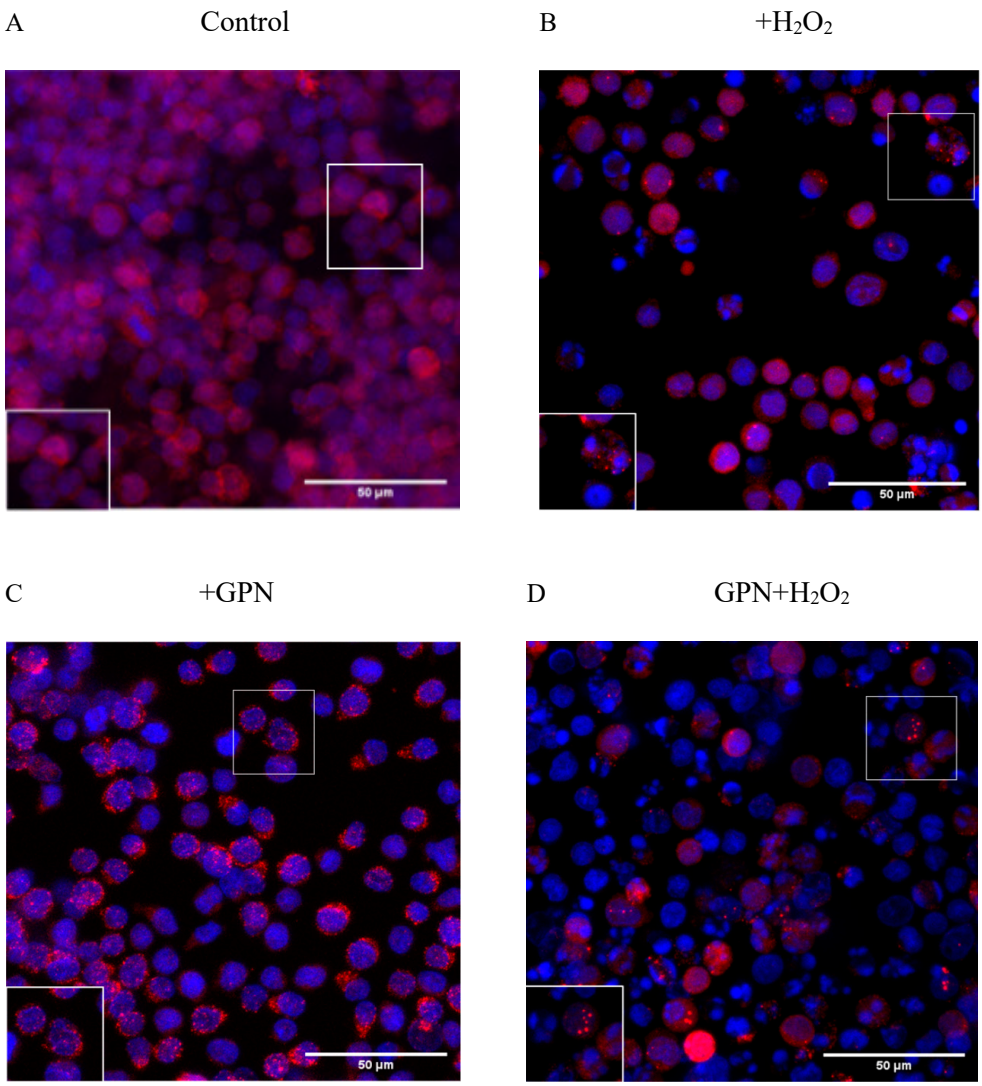


Figure 6 Visualization of galectin-1 puncta indicating lysosomal leakage in leukemic U937 cells (Paper II).
Confocal laser scanning microscopy was used to visualize galectin-1 immunostaining as *red* puncta, in the cells treated with or without H_2O_2, and/or GPN. Nuclei were stained with DAPI *(blue)*. Scale bar, 50 µm. (**A**) Control cells (no treatment) **(B)** cells treated with H_2O_2, **(C**) Cells treated with GPN are shown as the mean from each image**, (D)** Cells treated with H_2O_2 and GPN.

2.2.4. Internalization of cystatin D and co-localization with cathepsin C

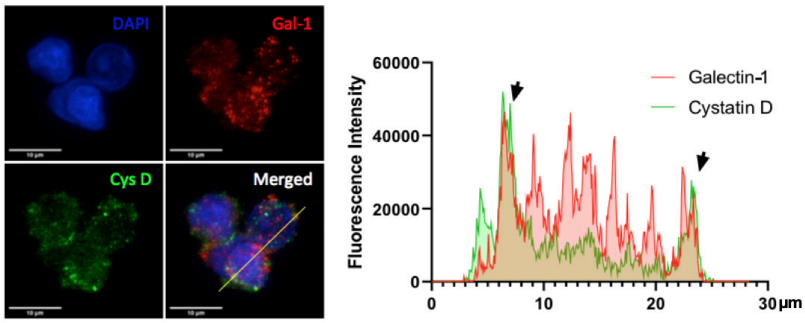
We next wanted to independently verify the intracellular co-localization, if it exists, of internalized cystatin D (the inhibitor), endogenous cathepsin C (the target enzyme) and internalized GPN (the substrate). For this purpose, we went after a galectin-1 and carbohydrate-binding dependent intracellular process that is independent from cathepsin C inhibition by internalized cystatin D (Figure 5). Keeping this in mind, as we have previously shown that cystatin D is taken up by the cells and retains in vesicles using CLSM **(**Figure 1).

Upon vesicle insult following the internalization and penetration of GPN into the lysosomes of U937 cells pre-treated with and without cystatin D and/or 40 μM H_2O_2. After 6 h of incubation, externally added GPN quickly accumulates inside lysosomes where it is hydrolyzed by the lysosomal enzyme cathepsin C, causing osmotic lysis (162). Co-localization of the large majority of GPN-induced galectin-1 puncta with internalized cystatin D (green) indicated that the galectin-1 accumulation (red), as expected was around damaged lysosomes which contain cathepsin C as shown in (Figure 7A). This provided a qualitative information on intracellular availability and activity of internalized cystatin D. Moreover, when observing and counting the galectin-1 accumulation into puncta in the cytosolic space of U937 cells using the image processing sofwares (Fiji-ImageJ and amarisoft) to give an automated unbiased quantitative analysis of the puncta. The total number of galectin-1 puncta and nuclei were quantified from numerous vision fields in all captured images. The results were expressed as the median number of galectin-1 puncta/nucleus: 2.92 (n = 30), 1.53 (n = 28), 2.92 (n = 26), and 0.58 (n = 21), after treatment with 0.3 mM (GPN, GPN+Cyst D, GPN+H_2O_2, and GPN+H_2O_2+Cyst D) respectively (Figure 7B).

There was a clear reduction of puncta formation, both for normal and H_2O_2 stressed U937 cells by external addition of cystatin D. It markedly decreased the number of GPN-induced galectin-1 puncta per nucleus, strongly indicating that cystatin D had indeed been internalized into the same compartment as cathepsin C and inhibited its enzyme activity. In addition, external addition of cystatin D resulted as well in decreased puncta formation after the cells were pretreated with H_2O_2, like the effect of cystatin D after addition to GPN-treated cells. These results gave a direct evidence that cystatin D indeed gets internalized in intact cells and reaches a compartment where its theoretical target enzyme (cathepsin C) resides. Moreover, it is obvious that the cystatin is functionally active as a cysteine protease inhibitor and the amount of cystatin D internalized is sufficiently high to down-regulate lysosomal cystatin C. The puncta assay provided evidence that cystatin D can inhibit lysosomal damage, when it is induced externally by the artificial substance, GPN. Of biological relevance for regulation of cell death, it also indicated that cystatin D may inhibit lysosomal damage in leukemic cells under oxidative stress induced by

H_2O_2. This finding suggests that cystatin D, if extracellularly added, might be able to save the cell from lysosomal damage and potential LCD caused by oxidative stress. A plethora of different enzymes and the molecules regulating them may translocate from the lysosomes to the cytosol when the lysosomal membrane is damaged. Clearly, more studies to understand how cystatins fit in the complex interplay within the protease/anti-protease web are needed, to explain the net effect of viability decrease by the externally added cystatins (119-120, 162).

A



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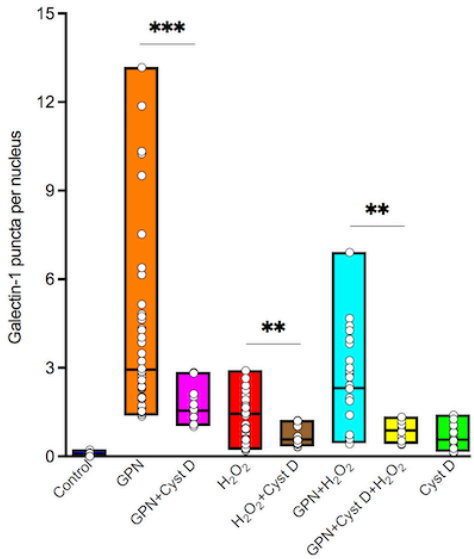

Figure 7 Confocal fluorescence microscopy of galectin-1 and cystatin D in leukemic U937 cells (Paper II).
(A) Confocal laser scanning microscopy was used to visualize galectin-1 (*red*) and cystatin D (*green*) immunostaining in the cells cultured in the presence of cystatin D (3μM) and treated with GPN. Nuclei were stained with DAPI *(blue)*. Scale bar, 10 μm. Profile of fluorescence intensity for each channel was plotted along the *yellow lines* shown in the right-hand part of the figure. Co-localization in some puncta is indicated by *black arrowheads*. **(B)** The number of puncta/nucleus in cells treated with H_2O_2, cystatin D and/or GPN are shown as the mean from each image analysed (*white symbols)*. *Horisontal lines* in the floating bars represent the median of all values from 4 independent experiments. Groups of data from combined experiments (12-45 images) were compared by Mann-Whitney test and the significance is indicated in the figure (**p<0.01, ***p<0.001).

*2.1.3. Cystatin D morphologically changed melanoma cells by DHM (**Paper IV**)*

In Papers I and Paper II, we observed that externally added human cystatin D affected cell death in U937 leukemic cells induced by oxidative stress including H_2O_2 by enhancing apoptosis (Paper I), but inhibited LCD (Paper II) using end-point methods. To attempt to further study cystatin D effects on cancer cell death mechanisms, we used digital holographic microscopy (DHM) as a method to address the possibility that cystatin D could affect cell death in cells derived from solid tumors. DHM can be used for analysis of an entire cell population or individual cells. Holograms are captured after treatment at certain time-points, or endpoint only. Captured holographic images can then be used to observe changes in morphology, cell number, movement, and division over time or between control and treated cells (163-164). Human recombinant cystatin D was externally added in the presence of a rather low concentration of H_2O_2 (50 μM), to catch early signs of apoptosis in human melanoma A375 cells and possibly visualize the cystatin effects on morphological changes such as cell area and optical thickness at selected time-points. Preliminary results indicated a reduced cell number in cultures with cystatin D and H_2O_2, that was apparent in the captured holograms compared to control cells after 24 and 48 h. After 48 h, the cystatin D and H_2O_2 treated cells displayed obvious morphological changes, in that almost all the cells lost their original elongated epithelial shape, looked big and bloated, and stopped to proliferate, but without obvious signs of increasing cell death during the entire 48 h. In contrast, cells cultured with H_2O_2 only looked condensed with a smaller area and high thickness without normal cell protrusions and with visible gaps among cells after 24 h. Most cells in the population incubated with standard medium with no treatment (control cells) looked healthy with respect to area and thickness and continued to proliferate throughout the entire experiment (Figure 8). This experiment gave no evidence that cystatin D seriously affects cell death in melanoma cells, neither by increasing nor decreasing it. However, the experiment should ideally be repeated with a series of gradually increasing H_2O_2 concentrations to find a level when cell death slowly appears under the 48 h study period, to optimize chances to see a possible cell death enhancing or inhibitory effects by cystatin D morphologically. The experiments conducted so far rather indicated that the main effects of cystatin D addition to A375 melanoma cells was to decrease their proliferation rate. Cystatin effects on proliferation rather than cell death is the topic of next section.

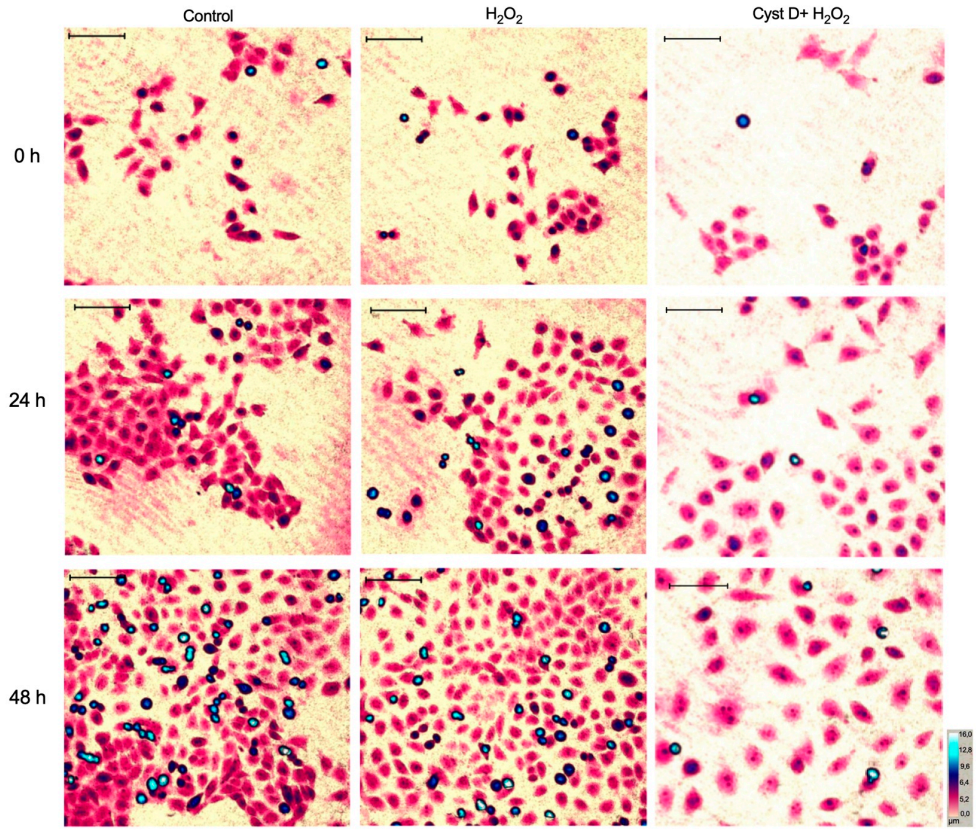

Figure 8 Morphology of A375 melanoma cells treated with cystatin D and H_2O_2 to induce cell death (Paper IV). The cells were incubated with and without 50µM H_2O_2 and/or 3 µM cystatin D. Digital holographic microscopy images were captured after 0, 24, and 48 h after treatment. Cell thickness is indicated by color-coding, as shown with the color bar to the right. Scale bars, 100 µm.

2.1. Cell growth

2.2.1 Cystatins effects on viable cell number

Our results had shown that cystatins C and D do not induce cell death alone (Paper I), but they can enhance apoptosis in U937 cells when they are simultaneously incubated with H_2O_2. This raised the question what the overall effects on cell number and viability are. To further investigate this, we used the MTT cell viability assay. Cells of three leukemic cell lines (HL-60, Jurkat and, U937) were seeded in standard medium, in the presence or absence of 3 µM of cystatin C or D and incubated for 48 h. The MTT result for control cells was set to 100% and the rest of

the results were related to it (Paper I). The viable cell count decreased in all three leukemic cells because of cystatin C addition and to a larger extent after cystatin D addition (Figure 9A). To further study the effect of cystatin C on viable cell count in other cell types, 1 or 5 µM concentration of recombinant human cystatin C was added to the medium of A375 melanoma, MCF-7 breast cancer, and PC-3 prostate cancer cell cultures for 24 h (following seeding and attachment of the cells for 24 h) (Paper III). Fewer viable cells were observed for A375 cells cultured in 1 µM cystatin C (53% of control) and when 5 µM cystatin C was used the number decreased further (43%). A similar dose-dependent decrease in viable cell number was also observed in MCF-7 and PC-3 cells (Figure 9B). We demonstrated a decrease in the number of viable cells which was paralleled by the internalization of cystatins C and D, indicating that down-regulation of intracellular proteolysis events is the cause of the effects observed (Figure 9).

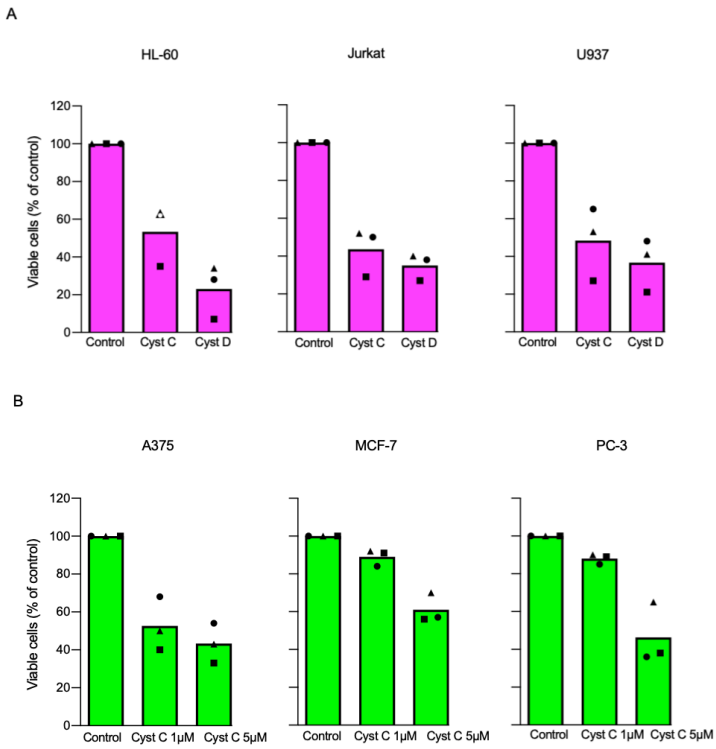

Figure 9 Effects of externally added cystatins on viable cell number in several cancer types (Papers I and III). **(A)** HL-60, Jurkat, and U937 cells were cultured under normal conditions and incubated with medium containing 0 (Control), Cyst C or Cyst D (3 µM) was externally added to the cell cultures for 48 h before analysis by MTT assay. **(B)** A375 melanoma, MCF-7 breast cancer, and PC-3 prostate cancer cells were incubated with medium containing 0 (Control), Cyst C 1 µM and Cyst C 5 µM for 24 h before running MTT assay. The experiments were made on three independent occasions, and 16–24 individual wells were analyzed for each condition, each time. Bars represent the mean result of the control cells in each experiment was set to 100%, and the rest of the values were correlated with that. Symbols represent mean results for each experiment (triangles, experiment 1; squares, experiment 2; circles, experiment 3). Statistics were calculated by Mann–Whitney test on groups of raw data.

*2.2.2 Effects of cystatin C on cell growth by DHM (**Paper III**)*

The studies of the effects of cystatins mentioned earlier on proliferation of cancer cells in culture using MTT assay were performed on a population level. However, studying single cancer cells behavior can be more informative than that in population-based data. CLSM can be challenging when running experiments for long time, bleaching or auto-fluorescence may occur. On the other hand, using DHM imaging allow live cell time-lapse imaging whereas no labelling or staining is needed. As the illumination intensity is very low, time dependent effects of cystatins on individual cells can be monitored for several days with no phototoxicity. The holograms acquired can be used later for cell division and proliferation studies, as well as cell morphology studies (151,163-167).

There are studies in the literature which have concluded that there is little or no effect on proliferation of human cells by cystatin C or D. To closely examine whether externally added cystatins C and D have the capability to affect cancer cell proliferation and, hence, potential to suppress tumour growth as previously shown by MTT results (Figure 9), we chose the melanoma A375 cells for in-depth studies. This was carried out as reduction in viable cell numbers by externally added cystatin C was the highest in these cells and, also, as their growth and behaviour properties are suitable for the DHM imaging method. A reduced A375 cell number in cultures with wild-type cystatin C was clear compared with control after 12 and 24 h (Paper III), but the cells looked healthy and normal with an elongated shape and cell protrusions. They continued to proliferate regularly each next to another - adherently attached to the bottom surface - throughout the entire experiment (Figure 10).

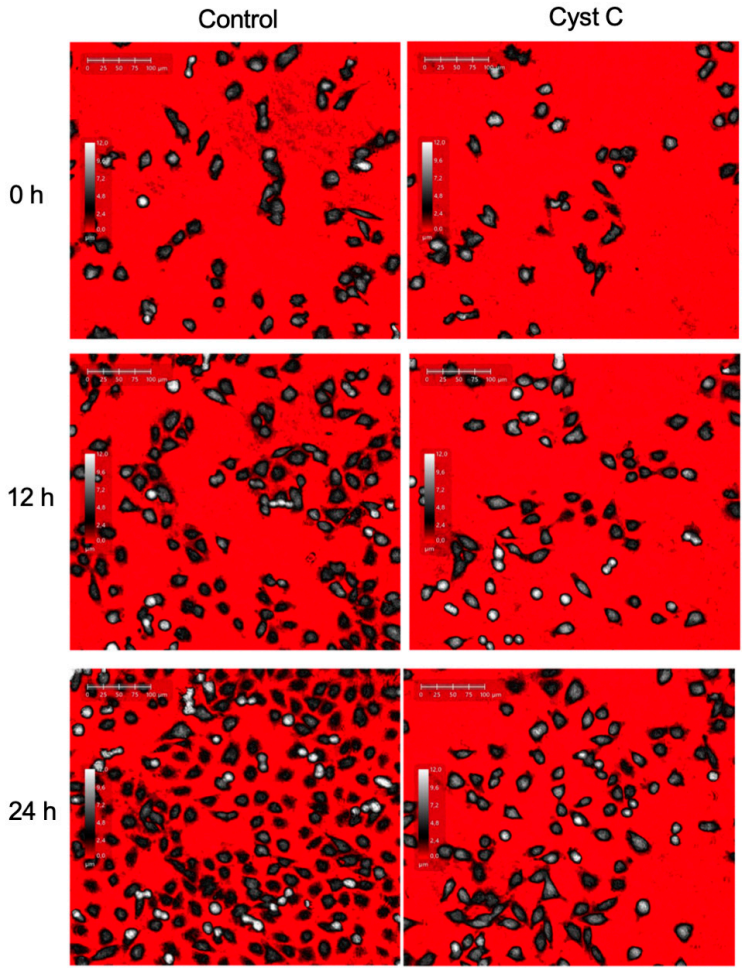

Figure 10 Morphology of melanoma A375 cells treated with or without cystatin C (Paper III).
The cells were incubated with or without cystatin C (5μM) and digital holographic microscopy images were captured after 0, 12, and 24 h. Cell thickness is shown by color-coding, as shown with the color bar to the right. Scalebars, 100 μm.

A trend toward decreased cell count in cultures incubated with wild-type cystatin C was seen in all experiments already after 12 h. This decrease in cell number in the presence of cystatin C could be due to either a decreased proliferation rate or increased cell death. For more detailed studies of the growth of A375 cell populations, we used mean cell count data of real-time holograms assessment and manually calculated the population growth rate and doubling time of A375 cell cultures with and without 5 μM wild-type cystatin C for 48 h (check methods chapter). Cells cultured in the presence of cystatin C had an increased doubling time

(20.1 h) compared with control cells (14.7 h) (Paper III). Moreover, cells incubated with wild-type cystatin C had longer cell division time, i.e., underwent fewer mitoses than control cells, as could clearly be seen in cell family trees (Figure 11).

To investigate whether cystatin internalization is a prerequisite for these observed effects on cell proliferation, we analyzed the two cystatin C variants, (R24A,R25A)- and W106F-cystatin C with different uptake properties (2, 30). Incubation with the variant W106F-cystatin C (with high cellular uptake rate) resulted in - already the first 12 h - fewer viable cells and a prolonged doubling time (24.0 h), but no effect was observed for (R24A,R25A)-cystatin C (low cellular uptake) compared to cells incubated with wild-type cystatin C (20.1 h). Thus, cystatin C causes longer cell cycle and division time leading to reduced proliferation of melanoma cells, and internalization seems to be a prerequisite for this effect (Paper III).

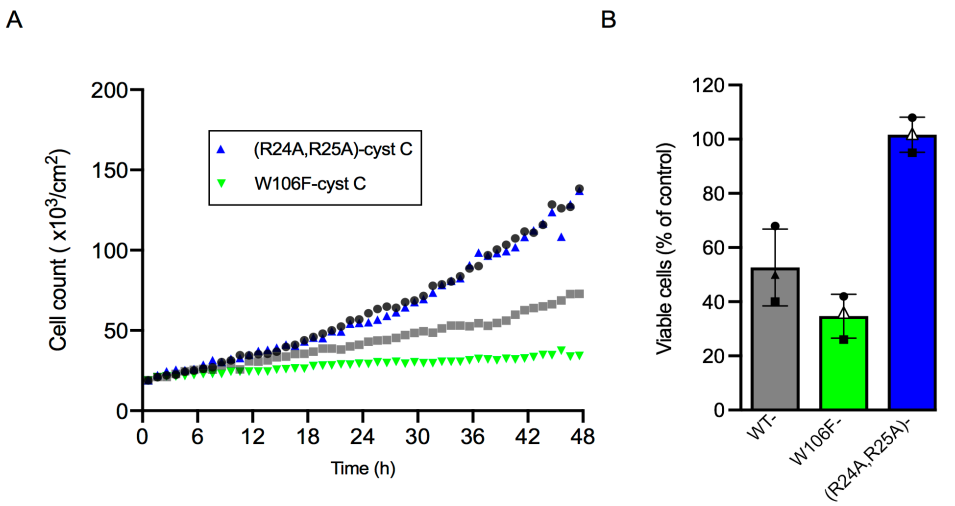

Figure 11 Effects of externally added cystatin C variants viable cell count in melanoma A375 cells (Paper III). The cells were incubated with medium containing 5 µM wild-type, W106F- or (R24A,R25A)-cystatin C and untreated control cells for 48 h. (**A**) Holograms were captured every hour. Mean growth curves based on normalized cell counts were drawn and compared to curves for no cystatin (blackcircles) and 5 µM wild-type cystatin C (gray squares). (B) End-point viable cell counts were measured by MTT assay for the 5 µM wild-type cystatin C and the two variants and compared to untreated control. Statistics using Mann-Whitney rank test were calculated on raw data. Bars represent the mean values, with error bars denoting SD. Circles, squares, and triangles represent the mean results from each experiment. distribution in all cells. The co-localization of the two cystatins was seen as yellow vesicles in the merged image of the red and green fluorescence shown below in Figure 1. Follow-up experiments of intracellular content and secretion of cystatin C and D in U937 cells were performed using ELISA, concluding that both cystatins are internalized in significant quantities. The co-localization results by CLSM in Figure 1, strongly indicate that cystatin D follows the same internalization route as cystatin C.

When A375 cells were treated with cystatin C, the cell cycle and division time increased gradually during the entire experiment in comparison to untreated control cells (Figure 12). This effect of cystatin C resembled that seen for salinomycin, a chemotherapeutic drug shown to increase cell cycle of dividing JIMT-1 cells (151).

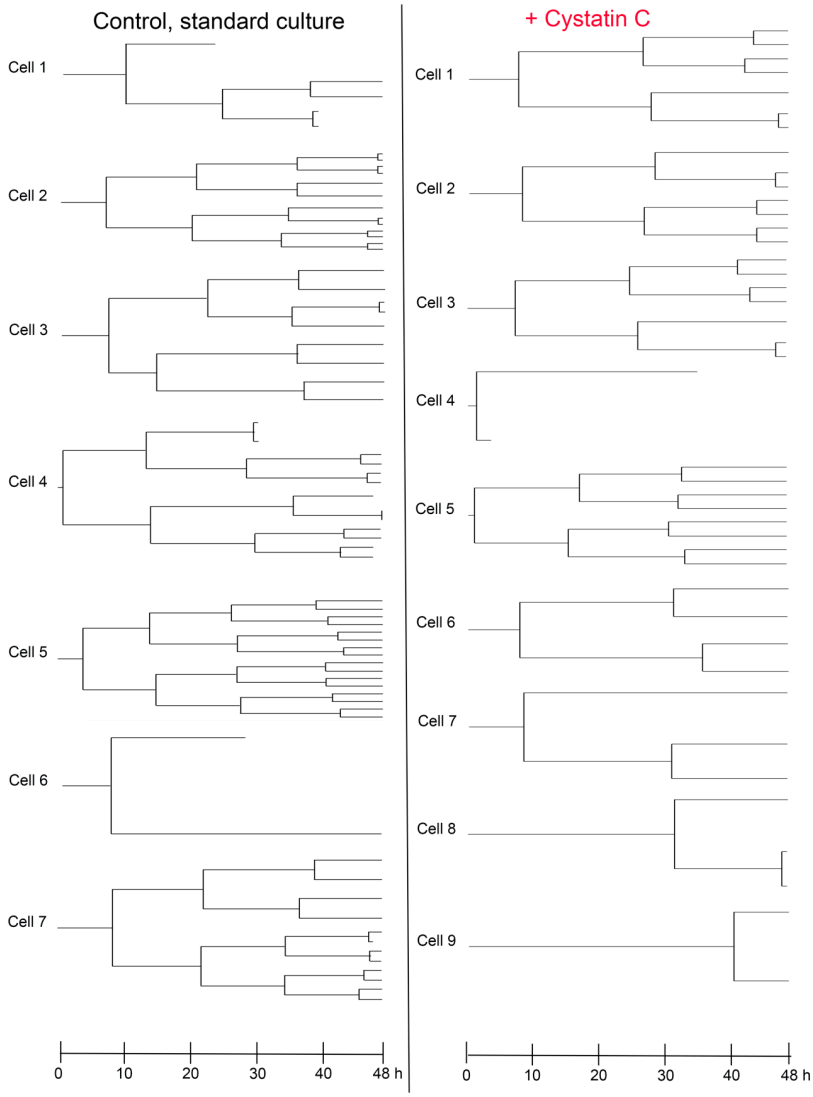

Figure 12 Family trees of melanoma A375 cells treated with or without cystatin C (Paper III).
The cells were incubated 0 (Control) or 5 µM cystatin C and holograms were captured every 5 min for 48 h. All cells in the first image were identified manually by the user and tracked until next division. The resulting daughter cells of each cell division were labeled, and tracking continued in later images. Data based on cell tracking were analyzed and presented as family trees. for control A375 cells grown in standard medium (left) or in presence of 5 µM cystatin C (right). Prolonged cell cycle and cell division time when the cells were cultured in the presence of cystatin C.

*2.2.3 Effects of cystatin D on cell growth of melanoma cells by DHM (**Paper IV**)*

Cystatin D was the most effective to boost cell death, and as effective as cystatins C to reduce cell proliferation to reduce proliferation in leukemia and melanoma cell cultures in (Paper I, Paper II). In Paper III, cystatin C reduced the proliferation of A375 melanoma cells by increasing cell cycle time of diving cells. Hereby, we wanted to evaluate the relative effects of externally added cystatin D on the growth of A375 melanoma cells.

Holograms of A375 cells were captured at time intervals of 0, 24 and 48 h. The control (untreated) cells were adherently attached to the bottom surface and grew regularly each next to another. Moreover, the cell optical thickness seemed relatively homogenous among the cells (visualized by pink colour in Figure 12). The cells treated with externally added cystatin D had visibly increased area (cells looked flat and bloated) after 24 h, and their optical thickness decreased in comparison to the untreated cells. The next 24 h caused severe changes in the cell morphological characteristics as the edges of the cells seemed to disappear and the decreased number of cells was prominent in a time-dependent manner. The cells with a dense, sharp blue rise in height, are either mitotic or apoptotic cells (Figure 13).

It has been reported that cystatin D is located within the cell nucleus at specific active chromatin sites and modulates gene transcription as well as cystatin D protein expression in human colon cancer cells, whose anchorage-independent growth and migration is then inhibited (168). Furthermore, cystatin D gene (*CST5*) knockdown abolished the anti-proliferative effect of the active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$). Therefore, *CST5* is proposed as a tumor suppressor by contributing to the anti-cancer action of 1α,25$(OH)_2D_3$ in human colorectal cancer cells (169). Apparently, cystatin D is as effective as cystatin C in affecting cell behavior and properties.

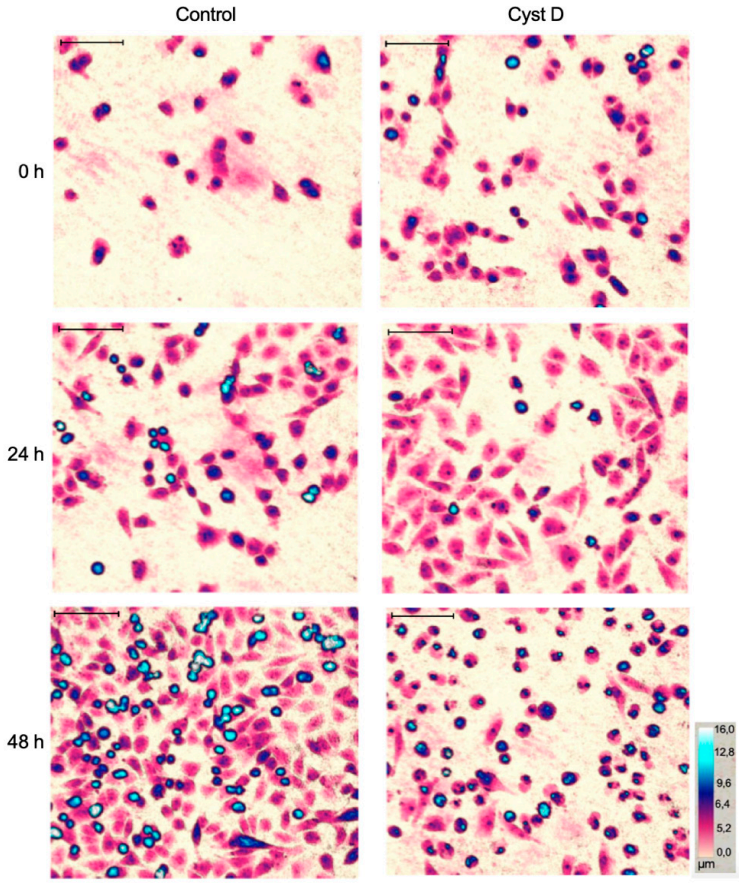

Figure 13 Morphology of melanoma A375 cells treated with or without cystatin D (Paper IV).
The cells were incubated with or without cystatin D (3µM) and digital holographic microscopy images were captured after 0, 24, and 48 h. Cell thickness is shown by color-coding, as shown with the color bar to the right. Scalebars, 100 µm.

Conclusions

A careful elucidation of the effects of cystatins on cultured cells is of a fundamental importance for the consideration of using secreted cysteine proteases inhibitors, cystatins, as natural biological anti-cancer drugs. By identifying such effects in studies of relatively well-defined and homogenous established cell lines, we could find clues to new individualized methods in cancer therapy. From a biological perspective, it is crucial to bear in mind that the cystatins we have studied (predominantly cystatins C and D) were added to culture media at physiological concentrations because they are alike those that can be found in human body fluids.

In all four papers included in this thesis, we have studied whether the net growth and survival of cells (of the A375, HL-60, Jurkat, MCF-7, PC-3 and U937 cell lines) in culture are affected by external addition of cystatins. Once we saw such effects by some cystatins, we also aimed to understand the mechanism behind these effects; if it was due to their known function as lysosomal cysteine cathepsin inhibitors or maybe by some other, proteolysis-independent mechanism. In Papers I and II, various experiments were carried out, mainly using leukemic U937 cells, under several conditions such as in hypoxia, normoxia, and under treatment with H_2O_2 or GPN, with different cystatins added to the cell cultures or not. This was done to determine which of the cystatins and cathepsins are involved in the affected cell death and/or growth processes.

We concluded that cystatins D and C had the most prominent effects. These cystatins can reduce cell proliferation by themselves in addition to enhancing apoptosis induced by oxidative stress. Their internalization paralleled such effects on U937 cells. Immunoblotting assays showed that the internalized cystatin D was non-degraded and, therefore, likely still biologically functional as a cysteine protease inhibitor inside the cell. Furthermore, the results from confocal laser scanning microscopy demonstrated that the internalization of cystatin D indeed occurs, leading to its localization in endo-lysosomal vesicles. Such an uptake process is prominent in all three leukemia cell lines studied. There was a partial co-localization of internalized cystatin D with cathepsin C in GPN-damaged lysosomes of U937 cells according to galectin-1 puncta assay, and cathepsin C activity in cellular homogenates was inhibited in parallel to anti-proliferative effects by cystatin D externally added to these cells.

In Papers I and II, we obtained end-point results at the cell population level. Still, we could not relate the results to cystatin effects on a specific cellular sub-population or individual cells. To solve this, we performed time-lapse kinetic cell proliferation assays for up to 48 h in Paper III using digital holographic microscopy. We could extract live 3D images and cell tracking data for individual cells in the cultures. Since the power of the laser light used is low, and no staining or labelling is needed, this time-lapse live cell imaging technique is considered non-invasive. It can be used for imaging over long-time intervals as well as population-based end-point assays.

In Paper III, we found that internalized cystatin C decreased proliferation of melanoma A375 cells because of rendered cell cycle and division times. Leading to increased doubling times, yet cells treated with cystatin C were like control cells regarding morphology characteristics and healthy appearance. This anti-proliferative effect of cystatin C was evident already after the first 12 h of treatment. The use of two cystatin variants, W106F-cystatin C (with high cellular uptake rate) and (R24A,R25A)-cystatin C (with low cellular uptake rate), in the same set of experiments strongly indicated that cellular internalization is a prerequisite for this effect. Moreover, a similar dose-dependent reduction in viable cell numbers as was observed when cystatin C was added to A375 cell cultures, was also seen for the two other cell lines originating from solid tumors in Paper III, indicating that this effect is not unique for melanoma A375 cells.

In Paper IV we used digital holographic microscopy to investigate differences between control and cystatin D treated cells in the presence and absence of H_2O_2 to induce cell death in A375 cells. We hypothesized that there would be morphological differences among cells after the external addition of cystatin D and that cell proliferation would be downregulated. Interestingly, we found morphological differences among cells incubated with and without cystatin D similarly as cystatin C in Paper III and that cystatin D, like cystatin C, can reduce cell proliferation of melanoma cells.

In short, the main conclusions from this thesis work are:

I. Cystatins C and D can enhance cell death in leukemic cancer cells during oxidative stress
II. Cathepsin C has been identified as a main target enzyme which is inhibited by internalized cystatin D in leukemia cells
III. Capturing of live time-lapse 3D images and cell tracking data by digital holographic microscopy to analyze the behavior of individual cells incubated with cystatins provided a new, fruitful way to study cell cycle effects of the cystatins
IV. Externally administered cystatin D can inhibit lysosomal leakage, which is the hallmark of lysosomal cell death.

V. Cystatins D and C can reduce the proliferation of melanoma cells as well as leukemic cells, by slowing down the cell cycle

VI. Overall, externally added cystatins C and D can reduce the population growth of cancer cells both by:
   a. Augmenting cell death in stressed cells
   b. Having a direct anti-proliferative effect by delaying cell division

Future perspectives

Our studies of the cellular effects of externally added cystatins have demonstrated that especially C and D can affect death and growth of different cancer cell types. The studies have, furthermore, indicated that intracellular inhibition of cathepsin C by these cystatins is part of the explanation for such effects. It remains, however, to explain how cathepsin C is involved in the cellular pathways normally regulating cell death and cell proliferation, as well as the detailed mechanism leading to cystatin internalization. To pinpoint which receptor(s) that are involved in the cystatin uptake mechanism could be one approach to possibly allow pharmacological manipulation, to increase the uptake of the cystatins normally existing in the body and be beneficial to combat cancer cell growth.

One interesting approach that could provide more data on cystatins' death/proliferation effects on cancerous and normal cells in the future, would be to combine fluorescence and digital holography in the same device, to be able to study the correlation between uptake of the cystatins and death/reduced proliferation in individual cells. A prototype for such equipment exists, which should soon be available in the market. Justified by the results obtained in this thesis project, further work along these and other lines is certainly merited to elucidate the extension, molecular mechanism, and biological importance of the phenomenon of cystatins' ability to regulate cell death and proliferation.

Popular science summary

Naturally existing proteins in our bodies called cystatins, could be useful in cancer therapy

In the seventies and eighties, our laboratory described a protein in our bodies that was later named cystatin C. Today, we know that cystatin C is one member of a family of 12 similar proteins. Most of them are mainly found outside the cells of our bodies, in various body fluids, including plasma, urine, tears, saliva, brain fluid, milk and semen. These proteins are called type 2 cystatins (cystatin C, D, E/M, F, G, S, SN and SA). All cystatins can block and thereby inhibit the activity of a type of protein-degrading enzymes called cysteine proteases. Such enzymes are used in the body for example, when food containing proteins is broken down and when a bone is re-formed or in the healing process when bones are broken. However, the activity of these enzymes is uncontrolled in disease conditions such as cancer, inflammation, and heart diseases. Therefore, cystatins can regulate body processes both in health and disease. Of the 12 cystatins, cystatin C is the most studied one. As it is present in the blood its level increases when the kidney is not working well, which is a sign for a kidney disease, it can be determined by a simple laboratory test. On the other hand, cystatin D is a less known cystatin and is normally found in saliva and tears only.

Hundreds of research articles have been published over the last four decades to discover what cystatins do in our bodies, but we still do not know the answer. How do they exactly work? Can they be used to treat diseases if we give them as drugs?

To try to answer these questions, we have made several experiments in our laboratory using different research methods to see what happens when cystatins are added to melanoma, breast, prostate, or leukemia cancer cells. We found that cystatins C and D can increase the death of these cancer cells. We also saw that the living cells divided more slowly. Both effects resulted in reduced growth of the leukemia cells and pointed to a promising anti-cancer effect of the cystatins. Moreover, our results showed that the way they do this seems to be by entering the cells and finding their way to a compartment inside the cell near the nucleus called the lysosome, where they meet and inactivate an enzyme called cathepsin C.

Using a new technique called 3D holographic microscopy, we could study single cells and see that internalized cystatins C and cystatin D reduced the growth rate of

melanoma cancer cells. They evidently do this by slowing down the cell cycle so that cell divisions take longer.

Thus, from the studies we have done on cultures of cancer cells, it seems like cystatins could potentially reduce the metastasis and spread of cancer in the body. It is too early to state that cystatins can be used as drugs, still, the results we have obtained tell that it is an interesting possibility that they could be used as natural remedies to complement established cancer treatments.

Populärvetenskaplig sammanfattning

Naturligtvis existerande proteiner i våra kroppar som kallas cystatiner, kan vara användbara vid cancerterapi

På åttiotalet beskrev vårt laboratorium ett protein i våra kroppar som senare fick namnet cystatin C. Idag vet vi att cystatin C är en medlem i en familj på 12 liknande proteiner. De huvudsakligen extracellulära närvarande i olika kroppsvätskor inklusive plasma, urin, tårar, saliv, cerebrospinalvätska och spermier är typ 2-cystatiner (C, D, E / M, F, G, S, SN och SA). Alla cystatiner kan blockera och därigenom hämma aktiviteten hos en typ av proteinnedbrytande enzymer som kallas cysteinproteaser. Sådana enzymer används i kroppen till exempel när mat som På 70- och 80-talet beskrevs ett protein i vår kropp som senare fick namnet cystatin C. Idag vet vi att cystatin C är en medlem i en familj med 12 liknande proteiner. De flesta av dem finns främst utanför cellerna vår kropp består av, i olika kroppsvätskor som blod, urin, tårar, saliv, hjärnvätska, mjölk och sperma. Alla cystatiner kan blockera och därigenom hämma aktiviteten hos en typ av protein-nedbrytande enzymer som kallas cystein-proteaser. Sådana enzymer används i kroppen till exempel när mat som innehåller proteiner bryts ner och när ben återbildas eller i läkningsprocessen när ben bryts. Emellertid är aktiviteten hos dessa enzymer okontrollerad vid sjukdomstillstånd som cancer, inflammation och hjärtsjukdomar. Därför kan cystatiner reglera kroppsprocesser både vid hälsa och sjukdom. Av de 12 cystatinerna är cystatin C den bäst studerade. Eftersom den finns i blodet ökar dess nivå när njurarna inte fungerar, vilket är ett tecken på njursjukdom som kan fastställas genom ett enkelt laboratorietest. Cystatin D, å andra sidan, är en mindre känd cystatin som normalt bara kan hittas i saliv och tårar.

Hundratals forskningsartiklar har publicerats under de senaste fyra decennierna i ett försök att upptäcka vad cystatiner verkligen gör i våra kroppar, men vi vet fortfarande inte svaret. Hur fungerar de exakt? Kan de användas för att behandla sjukdomar om vi ger dem som läkemedel?

För att försöka besvara dessa frågor har vi genomfört ett antal experiment i vårt laboratorium med hjälp av olika forskningsmetoder, för att se vad som händer när cystatiner sätts till melanom-, bröst-, prostata- och leukemi-cancerceller. Vi fann att cystatinerna C och D kan öka döden när dessa cancerceller ät stressade och vi såg dessutom att de gjorde att cellerna delade sig mer sällan. Båda effekterna resulterade

i minskad tillväxt av cancercellerna och pekade på en lovande anti-cancereffekt av cystatinerna. Dessutom visade våra resultat att sättet cystatinerna gör detta på verkar vara genom att ta sig in i cellerna till en rum inne i cellen nära kärnan som kallas lysosomen, där de möter och inaktiverar ett enzym som kallas cathepsin C.

Genom att använda en ny teknik som kallas 3D holografisk mikroskopi kunde vi studera enstaka levande celler och se att cystatin C och cystatin D som tagit sig in i melanom-cancerceller minskade deras tillväxt-takt. De gör uppenbarligen detta genom att bromsa cellcykeln så att celldelningarna tar längre tid.

Således verkar det, från de studier vi har gjort på cancerceller odlade i laboratoriet, som om cystatiner kan ha potential att minska tillväxten och spridningen av cancer i kroppen. Det är för tidigt att säga att cystatiner kan användas som läkemedel, men de resultat vi har fått antyder att de möjligen kan användas som naturläkemedel för att komplettera etablerade cancerbehandlingar.

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