Regulation of legumain in human cells

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LIST OF PUBLICATIONS

Paper I

Smith R, Johansen HT, Nilsen H, Haugen MH, Pettersen SJ, Mælandsmo GM, Abrahamson M, Solberg R. Intra- and extracellular regulation of activity and processing of legumain by cystatin E/M. Biochimie. 2012 Dec; 94(12): 2590-9

Paper II

Solberg R, Smith RL, Almlöf M, Tewolde E, Nilsen H, and Johansen HT. Legumain expression, activity and secretion are increased during monocyte-to-macrophage differentiation and inhibited by atorvastatin. Submitted.

Paper III

Smith R, Solberg R, Jacobsen LL, Voreland AL, Rustan A, Thoresen GH, and Johansen HT. Simvastatin inhibits glucose metabolism and legumain activity in human myotubes. PLoS ONE. 2014 Jan 8;9(1):e85721

Paper IV

Smith RL*, Åstrand OAH*, Nguyen LM, Elvestrand T, Hagelin G, Solberg R, Johansen HT, Rongved P. Synthesis of a novel legumain-cleavable colchicine prodrug with cell-specific toxicity. *Contributed equally. Bioorganic & Medicinal Chemistry. 2014 http://dx.doi.org/10.1016/j.bmc.2014.04.056. In Press.

ABBREVIATIONS

AEP	Asparaginyl endoprotease
AP	Activation peptide
Cat	Cathepsin
CK	Creatine kinase
CBS	Colchicine binding site
DTT	Dithiothreitol
E-64	Trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ER	Endoplasmatic reticulum
EST	Expressed sequence tag
Farnesyl-PP	Farnesyl pyrophosphate
FCS	Fetal calf serum
FDA	Food and Drug Administration
GAG	Glycosaminoglycan
Geranylgeranyl-PP	Geranylgeranyl pyrophosphate
GFR	Glomerulus filtration rate
HCCAA	Hereditary cystatin C amyloid angiopathy
HCT116	Colorectal cancer cell line (ATCC CCL-247)
HEK293 cells	Human embryonic kidney 293 cells (ATCC, CRL-1573)
HL	Hodgkin’s lymphoma
HMG-CoA	3-hydroxy-3-methyl-glutaryl-Coenzyme A
IHC	Immunohistochemistry
IL	Interleukin
IP	Immunoprecipitation
K_i	Inhibition constant
KO	Knock-out
LDL	Low-density lipoprotein
LOX-1	Lectin-like oxidized LDL-receptor 1
LSAM	Legumain stabilization and activity modulation

M1	Proinflammatory macrophages
M2	Immunomodulatory and tissue remodeling macrophages
M3CL cells	Legumain and cystatin E/M co-over-expressing HEK293 cells
M4C cells	Cystatin E/M over-expressing HEK293 cells
M38L cells	Legumain over-expressing HEK293 cells
mAb	Monoclonal antibody
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompability complex
MMP	Matrix metalloprotease
NC-IUBMB	Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
pAb	Polyclonal antibody
PI-3	Phosphatidylinositol 3
PLC-γ1	Phospholipase C gamma-1
PMA	Phorbol 12-myristate 13-acetate
RAW264.7	Mouse macrophage cell line (ATCC, TIB-71)
RGD	Arginyl-glycyl-aspartic acid
RNAi	RNA interference
sALCL	Systemic anaplastic large cell lymphoma
SDS	Sodium dodecyl sulfate
SR-A	Scavenger receptor A
SW620 cells	Colorectal cancer cell line (ATCC, CCL-227)
TAMs	Tumor associated macrophages
THP-1	Human leukemia monocytic cell line (ATCC, TIB-202)
TGF-β	Transforming growth factor-β
TLR9	Toll-like receptor 9
TNF	Tumor-necrosis factor
TNFRI	TNF receptor I
TRAIL-R1	TNF-related apoptosis-inducing ligand-receptor 1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
uPA	Urokinase-type plasminogen activator
3D	Three-dimensional

ABSTRACT

The studies presented in this thesis aimed to investigate the regulation of the cysteine protease legumain in various human cells (**paper I-IV**), effects of statins (**paper II and III**) and a legumain-cleavable colchicine prodrug (**paper IV**). Legumain is the only known asparaginyl endoprotease and shows high structural similarities to the caspases. Legumain is reported to participate in extracellular matrix degradation by processing of pro-matrix metalloprotase-2, fibronectin, cathepsin B and L. The subcellular localization of legumain is thought to be mainly lysosomal, and dysregulation of legumain is suggested to be of consequence for proteolytic degradation and tissue remodeling. High legumain expression is correlated with cancer progression and malignancy as well as atherosclerotic plaque rupture, and pharmacological intervention targeting legumain may be useful.

In the present work, legumain and/or cystatin E/M over-expressing HEK293 cells were established, and used to study regulation, subcellular localization and interplay of legumain and cystatin E/M (**paper I**). Legumain over-expressing HEK293 cells secreted prolegumain that was able to be internalized and subsequently activated intracellularly (**paper I**). Cystatin E/M (the most potent endogenous inhibitor of legumain) was also secreted and internalized, and thus able to regulate legumain activity both intra- and extracellularly (**paper I**). Macrophages are key regulators in cancer and atherosclerosis. In **paper II**, legumain expression, activity and secretion were highly up-regulated during monocyte-to-macrophage differentiation (**paper II**). Interestingly, 90 % of the legumain produced in M-CSF-stimulated macrophages was secreted as prolegumain, and surprisingly legumain was detected in human sera (**paper II**). Secreted legumain points to extracellular functions, and quantification may have diagnostic or prognostic value.

Statins (HMG-CoA reductase inhibitors) have so-called pleiotropic effects beyond lowering the serum cholesterol level. These drugs are generally well tolerated, although skeletal muscle side effects are known, especially by simvastatin. In monocytes from patients treated with atorvastatin, legumain mRNA is shown to be down-regulated. Herein, statins were shown to inhibited legumain activity, expression, processing and secretion in various human cells types, such as macrophages (**paper II**), myotubes (**paper III**), and in legumain over-expressing HEK293 cells (Fig. 8), probably by interfering with the intracellular vesicular trafficking or by increasing the pH of the lysosomes (**paper III**). Legumain inhibition may contribute to the pleiotropic effects of statins.

A novel pharmacological strategy is to construct a prodrug of a cytotoxic drug conjugated with a legumain-cleavable peptide (Ala-Ala-Asn), which upon cleavage by legumain becomes cytotoxic to legumain over-expressing cancer cells and tissues. In **paper IV**, a novel legumain-cleavable colchicine prodrug (Suc-Ala-Ala-Asn-Val-colchicine) were synthesized and showed cell-specific toxicity towards cells expressing active legumain (legumain over-expressing HEK293 cells and HCT116 colorectal cancer cell).

In conclusion, the studies presented in this thesis have contributed to new knowledge about regulation of the cysteine protease legumain. It has demonstrated that regulation of legumain occur both extra- and intracellular by cystatin E/M. Pharmacological inhibition of legumain by statins or by utilizing legumain to release cytotoxic colchicine from a legumain-cleavable prodrug may be of therapeutic value in preventing morbidity and mortality in cancer and atherosclerosis.

INTRODUCTION

Proteolytic enzymes

Synthesis and degradation of proteins are essential for life and proper cellular function. Approximately 2 % of the functional genes in the human genome encode for proteolytic enzymes (proteases, peptidases or proteinases) [1]. Proteolysis is cleavage of the polypeptide chain by irreversible hydrolysis (nucleophilic attack) of the peptide bonds within the substrate. Exoproteases cleave at the C- or N-terminal end of a polypeptide chain resulting in release of either a single peptide or dipeptide, whereas endoproteases cleave internally in the polypeptide chain. The database MEROPS classifies proteases based on their proteolytical mechanisms (classes), evolutionary relationship defined by their globular three-dimensional (3D) folding (clans), and their amino acid sequence homologies (families) [2]. Four major classes of mammalian proteases are identified; aspartate, cysteine, serine and metalloproteases (Fig. 1). For the serine and cysteine proteases a distinct amino acid serves as the nucleophile (serine and cysteine, respectively), whereas for the aspartate and metalloproteases a water molecule is responsible for the proteolysis. To perform proteolysis, both serine and cysteine proteases requires a second amino residue, a proton donor (histidine). In addition, many families of cysteine proteases (e.g. family C1, C2) also have an additional third catalytic residue to orientate the imidazolium ring of the histidine.

A protease clan is defined as a group of families for which there are indications of an evolutionary relationship despite the lack of significant similarities in the sequences. Such relationship comes from similar linear arrangements of active site residues in the sequences or from similar protein folds (secondary or tertiary structure, respectively). The clans are named with two letters; the first letter is given by the catalytic type and the second letter distinguishes the clans by alphabetic order (e.g. clan CA, CD; Fig. 1). In each clan one or more protease families are included. A set of homologous proteases with significant similarity in the amino acid sequences (primary structure) is referred to as a protease family. The nomenclature of a protease family is represented by a letter followed by a number. The letter represents the catalytic type of the protease, whereas the number distinguishes the families in a numbered order (e.g. C13, C14; Fig. 1) [2].

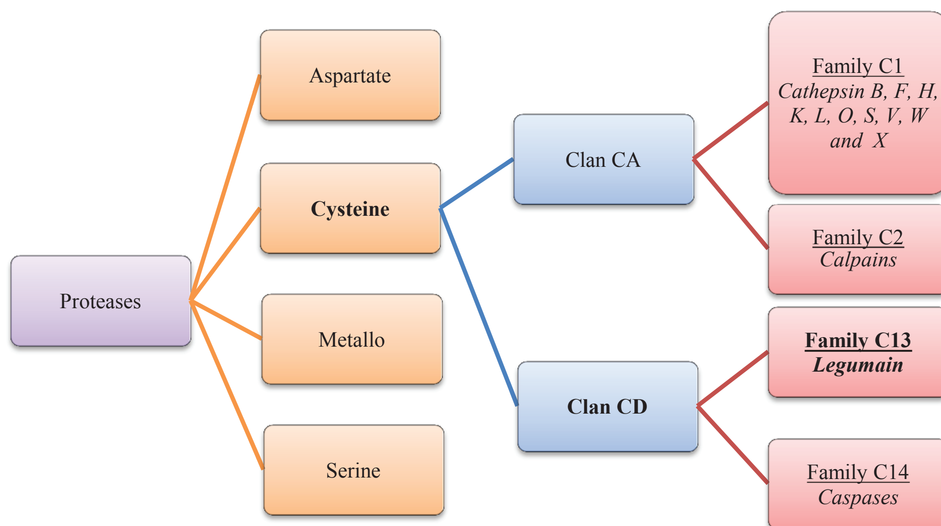

Figure 1. Classification of mammalian proteases according to MEROPS. Proteases are classified based on catalytic mechanisms and structure similarities. The proteases are first classified based on their catalytic type, hence aspartate, cysteine, metallo or serine (orange boxes). The proteases are further divided into clans (blue boxes) based on common evolutionary origins such as similar 3D protein structures or the order of amino acids comprising the catalytic part of the polypeptide chain. Each clan is further divided into one or more protease families (red boxes) based on high similarity in the amino acid sequences. Modified from [2].

Cysteine proteases

The first recognized cysteine protease was papain, found in the latex of the tropical papaya fruit. This enzyme is the archetype of the cysteine proteases and belongs to the clan CA, family C1. The mammalian analogs of papain are the cysteine cathepsins (cathepsin B, C (dipeptidyl-peptidase I), F, H, K, L, O, S, V, W and X; Fig. 1). Although most of the cathepsins are cysteine proteases, it is noteworthy to mention that cathepsin D and E are aspartic proteases, and cathepsin A and G are serine proteases [2]. In the following text, cathepsins refer to the cysteine cathepsins. In addition, the calpains, legumain and caspases are other cysteine proteases. The calpains belongs to the clan CA, family C2, and are dependent of a neutral environment, reducing conditions and calcium ions to acquire activity. Legumain (C13 family) and the caspases (C14 family) belong to clan CD, and will be addressed below.

The Schechter and Berger nomenclature model can be used to describe the interaction between the active site of an endoprotease and its peptide substrate [3]. The model divides the active site of the endoprotease into substrate-binding subsites (numbered S4, S3, S2, S1, S`1, S`2 and S`3) which binds the corresponding amino acids of the substrate (numbered P4, P3, P2, P1, P`1, P`2 and P`3), respectively [3]. The substrate-binding subsites are located on both sites of the catalytic site; the non-primed subsites are located on the N-terminal end of the substrate and the S1 subsite is nearest the catalytic site. The primed subsites are located on the C-terminal end of the substrate and S`1 is nearest the catalytic site.

The cysteine proteases are synthesized as inactive precursors (preproforms/proforms) and require a biochemical change such as hydrolysis to reveal its catalytic site. Hydrolysis of the inactive precursors can be facilitated either by other proteases or by auto-activation [4-6]. The precursors are normally cleaved in the endoplasmic reticulum (ER) and transported to the acidic endosomes/lysosomes and auto-activated. Auto-activation can therefore be regulated by post-translational modifications of cysteine proteases affecting the transport to the lysosomes. Interestingly, glycosaminoglycans (GAGs) have shown properties for accelerating the activation process of cysteine proteases [7-9]. Both the auto-activation and activity of cysteine proteases can be regulated by inhibitors such as the cystatins [10, 11] and the proteases require a reductive environment to achieve activity.

Cysteine cathepsins

The name "cathepsin" is from ancient Greek meaning degrade or "boil down" (kata, down; hepsein, boil). Cysteine cathepsins share a conserved active site which is formed by a catalytic triad consisting of cysteine, histidine and asparagine residues. Cathepsins have three well-defined substrate-binding subsites (S2, S1 and S1`) [12]. Cathepsin B represents the first described mammalian member of the C1 family and needs low pH for optimal activity (Table 1) [13]. Interestingly, some cathepsins also show activity in neutral environment [14, 15]. Most members of the cathepsins are endoproteases except dipeptidyl-peptidase I (previously named cathepsin C) and cathepsin X which are exoproteases. However, cathepsin B and H exert both exoprotease and endoprotease activities. The proteolytical active forms of cathepsin B, H and L are expressed as single and two-chain forms. The two-chain form is possibly due to intramolecular S-S-bridging. Most of the cathepsins are ubiquitously expressed and initially believed to be unspecific proteases in protein turnover. Many cathepsins share redundant substrate specificity, meaning that function loss of one particular enzyme does not necessarily lead to changes in cellular functions or a characteristic

phenotype. In gene knock-out (KO) studies both cathepsin B and L KO mice were born fertile. Cathepsin L KO mice show retarded hair growth and develop hair loss [16], and cathepsin B KO mice develop normally without any apparent neurological or behavioral deficits [17]. However, studies on specialized cells revealed predominantly expression of certain cathepsins. For example, cathepsin K seems to be important for bone remodeling and cathepsin K KO mice develop osteopetrosis, elevated numbers of osteoclasts, regions of disorganized bone microstructure and increased bone fragility [18]. Also, cathepsin S seems to be involved in antigen presentation since dendritic cells from cathepsin S KO mice have reduced cleavage of the invariant chain leading to decreased antigen presentation by the major histocompatibility complex (MHC) class II [19].

Legumain

Legumain, a lysosomal asparaginyl endoprotease (AEP), is well-conserved throughout evolution and belongs to the clan CD, family C13 of cysteine proteases (Fig. 1) [2]. It was first discovered in germinating bean cotyledons of kidney beans, which is a part of the plant embryo within the plant seed. Later, legumain has been identified in several other plant seeds [20-24]. The amino acid sequence of legumain from castor beans was shown to be homologous with an enzyme from the fluke *Schistosoma mansoni* [25], which later was reported to have AEP-activity [26]. The AEP discovered by Csoma & Polgár was named "legumain" by NC-IUBMB (1992) because of its abundance in seeds from the Fabeceae plant family (legume) [24]. In 1997 human expressed sequence tags (EST) were shown to be homologous with legumain from plants and *Schistosoma mansoni*, and human legumain cDNA was cloned [27]. The human legumain gene (*LGMN*) is localized on chromosome 14q32.1, and the legumain protein consists of 433 amino acids [27, 28]. The human legumain shares 30-35 % amino acid sequence homology with legumain from *Schistosoma mansoni* and plants [27].

Legumain isolated from pig kidney showed AEP-activity that was inhibited by ovocystatin (cystatin from chicken egg white) [27]. Legumain shows proteolytic activity for substrates rich on asparagine (Asn) in their side chains, and the activity is lost by blocking the thiol group (by e.g. iodoacetate), thus confirming that legumain is a cysteine protease [24]. Legumain is different from the cysteine cathepsins due to the strict requirement of one particular amino acid (asparagine, Asn) in the P1 position [24]. In contrast to the cathepsins, legumain do not have other well-defined substrate-binding surrounding subsites except the S1 site for Asn. What also distinguishes legumain from the C1 and C2 families of cysteine

proteases is the lack of inhibition by the cysteine protease inhibitor E-64 or leupeptin [22, 27]. The activity and stability of legumain are shown to be pH-dependent; legumain has a catalytic optimum at approximately pH 6 and is unstable at pH-values above 6 [27]. Initially, legumain was reported to have absolute substrate specificity towards Asn, however, legumain can also cleave after aspartate (Asp) residues at low pH, thus acquiring caspase-like properties [27, 29]. The catalytic dyad of legumain is shown to be His^{150} and Cys^{190} based on studies of mutant forms of the enzyme [30]. By investigating the catalytic dyad of legumain, it was proposed a similar folding of the catalytic site as the protease families of clostripains (family C11), caspases (family C14) and gingipain R (family C25), hence reflecting an evolutional relationship between these proteases [30]. In 2013 the first crystal structure of legumain was revealed and confirmed a 3D similarity (tertiary structure) to the caspases (Fig. 2A) [31, 32].

Mammalian legumain is expressed as a proform of 56 kDa. The 56 kDa proform consists of a catalytic domain and a C-terminal prodomain composed of an activation peptide (AP, Lys^{287}-Asn^{323}) and a legumain stabilization and activity modulation domain (LSAM) [31]. The catalytic domain consists of six-stranded β-sheets flanked by five major α-helices (caspase-like fold; Fig. 2A) [31, 32]. The catalytic site is stabilized by the LSAM due to electrostatic interactions. The catalytic domain has a highly negatively charged surface while the LSAM has a positively charged surface explaining the stabilization of prolegumain at neutral pH. In the acidic environment of the lysosomes legumain is auto-processed to a 47 kDa inactive and a 46 kDa active intermediate form [33]. Generation of the active 46 kDa form is thought to require cleavages of the proform at the C-terminal (Asn^{323}) and at the N-terminal (Asp^{25}) sides (Fig. 2B) [33]. However, studies of the crystal structure revealed that only cleavage at Asn^{323} is required to achieve fully active legumain [31]. Although cleavage at Asn^{323}, LSAM is believed to still be connected to the catalytic domain by electrostatic interactions at pH values above 4.5 [31]. Legumain can further be processed to an active mature form of 36 kDa, but this step is probably not autocatalytic and dependent on other proteases, e.g. the cathepsins (except cathepsin B) (Fig. 2B) [33, 34]. Recently, GAGs and alginates have been shown to accelerate auto-activation of prolegumain, possibly by electrostatic interference with the prodomain making the catalytic site more accessible [8, 35]. Also, legumain is postulated to have asparagine carboxyprotease activity [31].

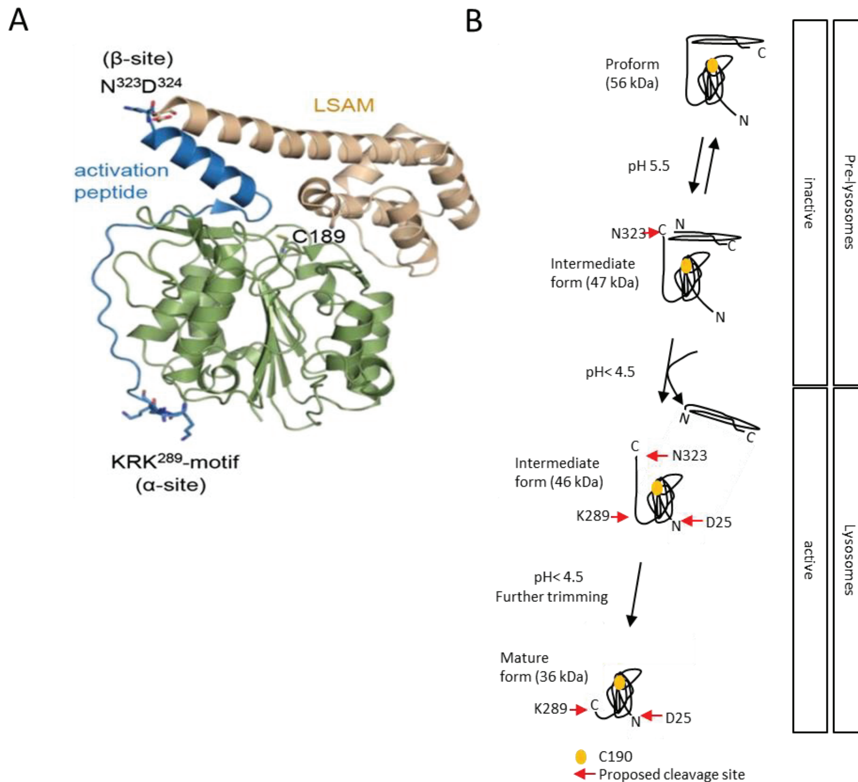

Figure 2. The crystal structure (A) and the proposed multistep auto-activation process (B) of human legumain. **A.** Legumain contains three different domains; the legumain stabilization and activity modulation domain (LSAM, wheat), the activation peptide (Lys^{287}-Asn^{323}, blue) and the catalytic domain (green). To gain full legumain activity, cleavages at Lys-Arg-Lys^{289} (α-site; KRK^{289}) and Asn-Asp^{324} (β-site; ND^{324}) are required. The catalytic cysteine residue (C189) is sheltered by LSAM. Adopted from [31]. **B.** An illustration of the proposed process for auto-activation of legumain. The inactive 56 kDa proform is present in prelysosomal compartments and converted to the inactive 47 kDa and the active 46 kDa intermediate forms by sequential autocatalytic cleavage at Asn^{323} and Asp^{25} in an acidic enviroment. The autocatalytic cleavage at Asn^{323} is reversible. Cleavage at Lys^{289} is required to allow dissociation of the prodomain and revealing of the active site. The active 46 kDa form is further trimmed to the mature active 36 kDa form. Adopted and modified from [31, 32].

Legumain is ubiquitously expressed in mammals with the highest expression in kidney, placenta, spleen, liver and testis [27, 36]. Although legumain is localized mainly to the lysosomes, translocation of legumain to other cell compartments (e.g. cell nuclei) and secretion to the tumor microenvironment have been reported [37-40]. Legumain have an Arg-

Gly-Asp[120] (RGD) motif which binds to the *αvβ* integrin receptor [37]. Upon binding to the *αvβ* integrin receptor, the pH optimum of legumain activity is increased, indicating that legumain also could be active in a slightly less acidic environment than the lysosomes [31].

Legumain KO mice are born viable and fertile with no distinct anatomical or morphological abnormalities, however, decreased growth rate is observed [41]. In wild-type mice, legumain is expressed in the apical endocytic compartments of the proximal tubule cells which are critical for reabsorption and degradation of proteins [42]. Indeed, legumain KO mice have impaired kidney function, increased plasma creatinine concentrations and decreased glomerulus filtration rate (GFR) [43]. Also, the legumain-deficient mice have enlarged lysosomes and impaired processing of the two-chain active forms of cathepsin B, H and L [41]. Interestingly, fibronectin is accumulated in the tubulointerstitial area of legumain KO cells and direct cleavage of fibronectin by legumain is shown *in vitro*, indicating a role of legumain in extracellular matrix (ECM) turnover [44]. In addition, legumain can activate proMMP2 *in vitro,* also contributing in ECM degradation [45].

Legumain-deficient dendritic cells show impaired signaling of the Toll-like receptor 9 (TLR9) due to reduced cleavage of the C-terminal segment of TLR9 [46]. Also, it seems that legumain has a role in processing of microbial tetanus toxin antigen for epitope presentation by MHC class II [47]. In addition, the initial step for removal of the invariant chain is performed by legumain and/or other proteases in B-lymphomas [48, 49]. However, in bone marrow-derived dendritic cells from legumain KO mice no difference was observed in MHC class II presentation compared to the wild-type littermates [50]. The C-terminal prodomain of legumain has been implicated as an inhibitor of osteoclast formation from macrophages, and thus bone resorption [51]. Legumain-deficient mice are characterized by significantly enhanced body temperature, progressive hepatosplenomegaly and cytopenia, which are symptoms of hemophagocytic syndrome/hemophagocytic lymphohistiocytosis [52]. Legumain is also suggested to have other functions such as annexin A2 cleavage [53], epidermal cornification of skin [54], degradation of SET in the granenzyme apoptotic pathway [55], proliferation of hepatocytes [56] and tau hyperphosphorylation in Alzheimer`s disease [57]. In atherosclerotic unstable plaques and tumors, legumain is over-expressed [37, 58, 59].

Cystatins

Cystatins are endogenous inhibitors of the cysteine proteases. The cystatins were first discovered in chicken egg white in the late 1960s [60]. Since then, 12 human cystatins have been described and divided into type 1, 2 and 3 cystatins [10]. The general inhibition mechanism of all cystatins is reversible, substrate competition and equimolar. The cystatins generally bind their target enzyme tightly in the nanomolar to picomolar range [61]. The cystatins are classified to the clan IH, family I25, according MEROPS [2]. However, a sub-classification of the three types is still used. The type 1 inhibitors, cystatin A and B (stefin A and B), are members of the subfamily I25A [2]. The cystatin A and B lack a signal peptide sequence and are without disulfide bonds and carbohydrate side-chains [62]. The type 2 cystatins, including cystatin C, D, E/M, F, G, S, SA and SN (MEROPS subfamily I25B [2]), are slightly larger than the type 1 cystatins due to their signal peptide sequence [62]. Only the type 2 cystatins are able to inhibit legumain whereas all of the cystatin members are capable of inhibiting the cathepsins. The third type of cystatins is the kininogens, including high-molecular-weight-kininogen and low-molecular-weight-kininogen, and are mainly found in the blood and synovial fluid [10, 63].

The type 2 cystatins

Legumain is inhibited by the endogenous type 2 cystatins; cystatin C (K_i=0.2 nM), E/M (K_i=0.0016 nM) and F (K_i=19 nM) [64]. The type 2 cystatins are small proteins with low molecular mass (approx. 15 kDa) and primarily secreted due to a secretory N-terminally signal-peptide sequence of approximately 27 amino acids. Cystatin C is the most thoroughly studied of the type 2 cystatins. The cathepsin-binding site of cystatins consist of a tripartite wedge-shaped structure complementary to the active site clefts of the cathepsins [65]. Surprisingly, the type 2 cystatins have an additional interacting site only for legumain, meaning that cystatin C is able to bind both cathepsin B and legumain at the same time in a 1:1:1 complex [64].

Cystatin C is found in all body fluids with the highest concentrations present in seminal plasma and cerebrospinal fluid [63]. Cystatin C can be used to measure GFR since the expression and secretion of cystatin C are relatively constant from most nucleated cells [66, 67]. It is believed that cystatin C is involved in pathological conditions such as rheumatoid arthritis, atherosclerosis, Alzheimer`s disease and cancers [68-71]. A mutation in the cystatin C gene (Leu^{68}) has been linked to the dominantly inherited cystatin C amyloid

angiopathy (HCCAA) discovered in a population on Iceland [72, 73]. HCCAA is caused by amyloid deposition due to the nonfunctional Leu^{68} variant of cystatin C in the cerebral and spinal arteries and arterioles, leading to serious brain damage and recurrent hemorrhagic stroke [74].

Cystatin E/M, the most potent inhibitor of legumain, have approximately 35 % amino acid sequence similarity to cystatin C. Cystatin E/M is expressed in a variety of human tissues, including heart, brain, placenta, lung, liver, pancreas, spleen, thymus, prostate, ovaries, small intestine and peripheral blood cells [75, 76]. In contrast to the other type 2 cystatins, cystatin E/M has two forms; one glycosylated (17 kDa) and one unglycosylated (14 kDa) [76]. The cystatin E/M gene (*CST6*) is located on chromosome 11, whereas the other type 2 cystatin genes are located on chromosome 20 [75, 76]. Cystatin E/M KO mice are associated with juvenile lethality and defective epidermal cornification, a disease recognized as human type 2 harlequin ichtyosis [77]. This defective cornification is due to involvement of uncontrolled legumain activity [54], and co-localization of cystatin E/M and legumain have been reported in hair follicles [78]. Cystatin E/M has often been described in relation to cancer as a potential tumor suppressor, and we and others have demonstrated that cystatin E/M inhibits invasion of human melanoma and oral carcinoma cells [76, 79, 80]. The expression of cystatin E/M is reduced in many cancer forms due to epigenetic silencing [81-83]. On the other hand, over-expression of cystatin E/M is reported in pancreatic cancer [84]. However, it is generally accepted that increased cysteine protease activities and decreased expressions of the type 2 cystatins (C, E/M, and F) are associated with cancer, resulting in a potential disturbed protease/inhibitor ratio contributing to aberrant cellular functions.

The role of cysteine proteases in diseases

Cysteine proteases in cancer

Cancer remains one of the leading causes of mortality in most developed countries with an estimated death rate of 7.6 million people each year [85]. Cancer is a complex multicausal disease which involves accumulation of genetic and/or epigenetic alterations in cells, resulting in expression of oncogenes and decreased expression of tumor suppressor genes. Both inherited and environmental factors are important in cancer development. The tumor cells abilities to form metastases and invade surrounding tissues characterize the malignant nature of cancer. The six characteristic hallmarks of malignant cancer are uncontrolled cell division, inflammations, alteration of the microenvironment, angiogenesis, invasion and metastasis

[86]. The matrix metallo- and serine proteases have been widely described in cancer progression. However, numerous studies also link cysteine proteases to the various aspects of cancer such as proliferation, invasion, apoptosis and metastasis [87]. In general, up-regulation of cysteine proteases can be associated with various aspects of cancer progression. Increased expression of cathepsin B, L and legumain are associated with a more aggressive tumor type and poor prognosis of breast, colon and ovary cancers, and in adenomas of the pituitary [59, 88-91]. Also, the presence of cysteine protease inhibitors correlates with a more favorable prognosis of various cancer forms [92-95]. Cathepsins are suggested to interfere with apoptosis, proliferation, cancer growth, invasive growth and metastasis, thereby promoting malignancy [87]. Furthermore, there is diversity in the expression of specific cysteine proteases in tumor cells and tumor-associated cells at different stages during cancer development (Fig. 3A). This might indicate that each enzyme could have distinct functions in various cell types of the tumor, the tumor microenvironment and the surrounding tissues (Fig. 3A) [87]. For example, legumain is found both in the malignant, endothelial and stromal cells as well as in tumor-associated macrophages (TAMs) [37, 96].

Recently, TAMs were suggested to represent the 7th hallmark of cancer [97], reflecting the importance of TAMs in cancer development. TAMs consist of a polarized M2 ($CD36^+$, $CD68^+$, $CD80^-$, $CD163^+$) macrophage population, representing immunomodulatory and tissue remodeling macrophages. Many observations indicate that TAMs perform protumoral functions such as promoting tumor growth, angiogenesis, metastasis and suppression of adaptive immunity in the tumor environment [96, 98]. TAMs have direct interactions with the tumor cells both in the tumor microenvironment and of the perivascular macrophage-assisted tumor cell intravasation [99]. The protumoral cytokines secreted by TAMs include interleukins (ILs) such as IL-10, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNFα) and MMPs [100, 101]. The presence of extensive TAMs infiltration is correlated with poor prognosis [102, 103]. In recent studies, anti-TAMs effects are shown to contribute to tumor suppression. For example, the antineoplastic agent trabectedin has a selective cytotoxic effect on TAMs, resulting in significantly reduced production of IL-6, monocyte chemotactic protein-1 (MCP-1) and tumor growth [104]. Interestingly, abundant expression of legumain is present in TAMs [38, 96].

Figure 3: Potential roles of cysteine proteases in tumor and tumor-associated cells. A. The diversity of cysteine protease expressions in tumor and tumor-associated cells are shown. Cat, cathepsin. Modified from [87]. **B.** Suggested extra- and intracellular functions of cysteine proteases in cancer progression. Modified from [105].

As mentioned, cysteine proteases are suggested to participate in cancer progression (Fig. 3B). The mechanisms behind the up-regulation of cysteine proteases in cancer are believed to be gene amplification, increased transcript variants, increased post-transcriptional stability and/or epigenetic methylation [87]. The cysteine cathepsins and legumain are mainly localized to lysosomes, however, in cancer the subcellular localization of cathepsins and/or

legumain are changed [106-108]. For example, legumain is secreted to the tumor microenvironment and is associated with the plasma membrane [106, 107]. Also, cathepsin B, L and legumain are reported to be present in the nucleus of cancer cells and suggested to participate in histone degradation [40, 109, 110]. Both direct and indirect degradation of ECM and cell adhesion proteins are extracellular effects of cysteine proteases in cancer progression [87, 105]. The suggested ECM protein substrates of cysteine proteases are laminin [111, 112], type IV collagen [111], tenascin C [113] and fibronectin [44]. Furthermore, the cysteine proteases can participate in proteolytic cascade activation [105]. Particularly, cathepsin B has been shown to activate pro-urokinase-type plasminogen activator (pro-uPA) and uPA is important in degradation of the tumor matrix and basal membranes, resulting in increased tumor progression by migration and invasion [114, 115]. Also, degradation of cell-adhesion proteins such as E-cadherin by cathepsins B, L and S is described, leading to increased disseminative ability of tumor cells [116]. In addition, intracellular participation of cysteine proteases in ECM degradation has been addressed. For example, intracellular degradation of collagen in the tumor, macrophages and fibroblasts by cathepsins has been observed [117-119], in agreement with the finding of the endocytic transmembrane glycoprotein uPA receptor-associated proteins which direct the collagen IV for lysosomal delivery and degradation [87].

Cysteine cathepsins are involved in intracellular functions such as apoptosis [120], a key process in cancer development and progression. Both cathepsins and calpains have recently been shown to trigger apoptosis by different pathways [120]. It is believed that increased permeabilization of the lysosomes result in translocation of cathepsins to the cytosol, resulting in cleavage of substrates involved in apoptosis. The induction of leaky lysosomes is probably both stimulus- and cell type-dependent. For example, generation of reactive oxidative species (ROS) causes lysosomal leakage by destabilizing the lysosomal membrane [120-122]. In cell free systems, cathepsin B-release from the lysosomes have been shown to be mediated by caspase-2 or -8. Release of cathepsins into the cytosol during apoptosis is involved in degradation of the antiapoptotic Bcl-2 family members, including the proteolytic activation of Bid and/or activation of caspases [123-125]. In hepatocytes, cathepsin B has shown to contribute to apoptosis by a caspase-8-dependent process [120, 126]. In addition, TNFα has shown to increase cytosolic cathepsin B and subsequently enhanced mitochondrial release of cytochrome C [127] and cathepsin B KO mice were more resistant to TNFα-induced apoptosis compared with their wild-type littermates [128]. Furthermore, cathepsin B has in cell free samples been reported to cause chromatin condensation, a morphological hallmark of

apoptosis [120]. There are also examples of direct processing of caspases by cathepsins. For example, cathepsin B is able to activate caspase-11 [129], whereas cathepsin L is able to activate caspase-3 *in vitro* [130]. Interestingly, a pro-survival role of legumain has been reported in the parasite *Blastocystis* [131]. Also, legumain has been shown to cleave and inactivate SET, an inhibitor of the DNAse NM23-H1 [55], leading to DNA cleavage and apoptosis. This novel pathway of apoptosis (granzyme pathway) is initiated by the cytotoxic T cells, and is including delivery of granules containing granzyme A and B to the target cells via perforins [132, 133].

Cysteine proteases in atherosclerosis

Atherosclerosis is a chronic inflammatory disease and a major cause of morbidity and mortality in industrialized countries. It is a complex disease characterized by atherosclerotic lesions containing excessive inflammation and lipid accumulation [134]. Typical findings are increased thickness of the artery wall, foam cell formation and hardening of the arteries. The atherosclerotic lesions are divided into stable and unstable plaques. The unstable plaques account for only 10-20 % of all lesions, but are responsible for 80-90 % of all acute clinical events [135]. In general, stable plaques tend to be asymptomatic and are usually rich in ECM and smooth muscle cells, whereas unstable plaque are rich in macrophages and macrophage-derived foam cells and only the fibrous cap separates the lesion from the arterial lumen [136]. The fibrous cap is vulnerable to rupture, which can result in formation of thrombus and eventually occlusion and/or thromboembolism. The pathology of atherosclerosis involves many different cells such as macrophages, T lymphocytes, endothelial cells, vascular smooth muscle cells (VSMC) as well as platelets [100, 137].

Macrophages are as mentioned important cells in regulation and development of atherosclerosis [100]. Monocytes from blood enter the intima through interaction with up-regulated adhesion molecules on the inflamed and activated endothelium, and differentiate to macrophages. Macrophages engulf modified lipoproteins and transform into foam cells constituting fatty streaks and later the fatty core of an atherosclerotic plaque. In addition to lipid accumulation, macrophages are inflammatory cells releasing a variety of inflammatory cytokines such as TNFα, IL-1 and CXCL8, linking the two hallmarks of atherosclerosis: lipids and inflammation [100]. The interaction between lipids and macrophages are mediated through scavenger receptors such as scavenger receptor A (SR-A), CD36 and lectin-like oxidized LDL receptor-1 (LOX-1), which all promote uptake and accumulation of lipids.

Differentiated macrophages have distinct characteristics. The M1 macrophages ($CD36^-$, $CD68^+$, $CD80^+$, $CD163^-$) produce inflammatory cytokines and act as cell killing effectors, whereas the M2 macrophages ($CD36^+$, $CD68^+$, $CD80^-$, $CD163^+$) moderate the inflammatory response and remove debris [138, 139]. In addition, M2 macrophages promotes angiogenesis and tissue remodeling due to release of the anti-inflammatory IL-10 and TGF-β [100]. Within the atherosclerotic plaque, increased levels of M1 macrophages are associated with accelerated atherogenesis and increasingly unstable lesions due to secretion of MCP-1, MMPs and stromelysin [138, 140]. In addition, macrophages also secrete active cathepsin B, L and S which directly or indirectly digest and weaken the fibrous cap and contribute to plaque instability [141]. Interestingly, increased mRNA-expression of MMP-9, cathepsin B and legumain has been observed in unstable plaques compared to stable plaques [58], suggesting a direct or indirect role of legumain in ECM degradation of the fibrous cap either by activating MMP-2 [45] or by direct cleavage of fibronectin [44]. The main contributor for increased legumain expression in plaques is probably the macrophages, since increased legumain was abundant co-localized with the macrophage marker CD68 within the unstable plaques [58].

Pharmacological interventions

Statins

Statins are cholesterol-lowering drugs prescribed worldwide to patients with hypercholesterolemia. Statins interfere with the *de novo* synthesis of cholesterol, resulting in decreased blood LDL-cholesterol and prevention of cardiovascular morbidity and mortality [142-144]. There are six statins marketed in Norway today; simvastatin, lovastatin, pravastatin, fluvastatin, atorvastatin and rosuvastatin. All these statins inhibit the rate-limiting enzyme HMG-CoA reductase in the cholesterol biosynthesis (Fig. 4) but have different lipophilicity, half-life and potency [145]. The statins are administered either as an inactive lactone form (simvastatin and lovastatin) or as an active acid form (atorvastatin, fluvastatin, rosuvastatin and pravastatin). There is equilibrium between the lactone and acid forms *in vivo* controlled by hydrolysis, β-oxidation and glucuronidation [146, 147]. Although statins down-regulate the cholesterol synthesis, the main effect on cholesterol is caused by a subsequent up-regulation of the *LDLR* gene [148]. Inhibition of HMG-CoA reductase by statins also prevents synthesis of other important isoprenoid intermediates of the cholesterol biosynthetic pathway

such as farnesyl pyrophosphate (farnesyl-PP) and geranylgeranyl pyrophosphate (feranylgeranyl-PP) (Fig. 4) [149]. These intermediates are functioning as lipid attachments for posttranslational modifications of a variety of proteins including small GTPases such as Ras, Rho, Rac and Rab [149, 150]. Thus, isoprenylations of proteins regulates the covalent attachment, subcellular localization and intracellular trafficking of proteins.

In clinical trials, the overall effects of statins in prevention of coronary heart disease appear to be greater than what might be expected from changes in the lipid levels, suggesting effects beyond the cholesterol-lowering [143, 144]. These so-called pleiotropic effects of statins are suggested to include effects on monocytes/macrophages, endothelial, vascular smooth muscular and stem cells.

Although statins are generally well-tolerated, side effects like myotoxicity have been reported; fatigue, myalgia, myositis and life-threatening rhabdomyolysis [151, 152]. All these conditions fall under the myotoxicity term and unfortunately all statins are associated with myotoxicity. The incidence of statin-induced myotoxicity in patient is approximately 1-5 % based on data from randomized clinical studies [153]. However, in observational studies as many as 9-20 % of the patients experienced some kind of muscle related side effects [154, 155]. Simvastatin has the highest incidence rate of rhabdomyolysis defined as creatine kinase (CK) levels greater than 10 times the upper normal limit [153]. However, patients still experience myotoxicity with no or slightly increased CK. The toxicity of statins is dose-dependent, and the inactive lactone forms are reported to be more toxic than the active acid form [156]. Acute application of statins to human muscle cells *in vitro* have been shown to trigger apoptosis, and it is suggested that this effect is mediated by activation of phospholipase C (PLC)-γ1 and phosphatidylinositol (PI-3 kinase) [157, 158]. Interestingly, this apoptotic process was prevented by adding mevalonate, but not when squalene epoxidase or the squalene synthase were inhibited [159]. This indicates that inhibition of isoprenoid compounds such as farnesyl-PP, geranylgeranyl-PP, ubiquinone and/or dolichol were responsible for apoptosis [160, 161]. Furthermore, activation of the cysteine proteases, calpains, caspase-3 and -9 were observed in cell death caused by statins [162]. Interestingly, combining treatment of statins with bicarbonate used to control intracellular pH, reduces the risk of apoptosis and consequently myotoxicity [163], suggesting that lysosomal enzymes in the acidic cellular compartments could be important in statin-induced myotoxicity.

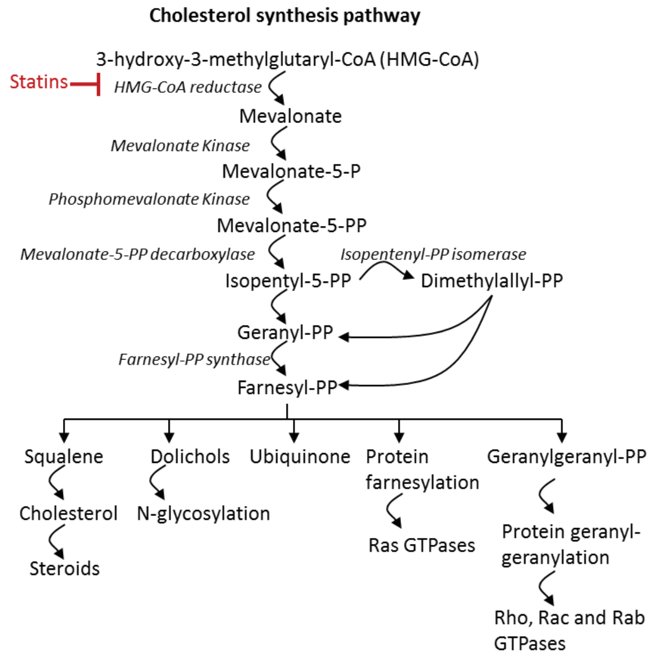

Figure 4: The cholesterol biosynthesis pathway. Statins block the rate-limiting step in the cholesterol synthesis by inhibiting the enzyme HMG-CoA reductase. Reduced activity of HMG-CoA reductase leads to decreased levels of downstream products such as cholesterol, dolichols, ubiquinone, and Ras-, Rho-, Rac-, Rab GTPase.

Drugs utilizing cysteine proteases

Pharmacological targeting of cysteine proteases such as the cathepsins and legumain could be beneficial since these are up-regulated in cancer and atherosclerosis. In general, inhibition of proteases has been a successful strategy in therapeutic interventions, including drugs inhibiting the angiotensin-converting enzyme and the human immunodeficiency virus protease. However, knowledge about functions, substrates, localizations and regulations of the cysteine proteases is insufficient to be able to anticipate the outcome of inhibiting one individual cysteine protease [14]. Potential problems with redundancy could also occur since target substrates could be cleaved by several enzymes not inhibited. Also, targeting the ubiquitously expressed enzymes such as cathepsin B and L could potentially be more hazardous compared to targeting enzyme with more specific and restricted expression such as cathepsin K and S. For example, inhibition of cathepsin K activity seems promising for the prevention of osteoporosis because of the prominent role of cathepsin K in osteoclasts and

bone remodeling [164, 165]. Indeed, odanacatib is a drug candidate for being the first cathepsin K inhibitor for prophylaxis of osteoporosis [166], and phase III clinical trials are currently ongoing. Also, targeting cathepsin S mainly localized in B-lymphocytes, macrophages and dendritic cells could be beneficial in halting development of autoimmune diseases such as myasthenia gravis and Sjögrens syndrome [167-169].

An alternative approach in drug development is to exploit the specific substrate cleavage site of an enzyme by conjugating a peptide sequence mimicking the substrate peptide cleavage site to a toxic compound or to an antibody which upon cleavage releases the toxic compound or exposes the antigen-binding site of the antibody [170-172]. Recently, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) accepted the use of brentuximab vedotin in relapsed or refractory Hodgkin's lymphoma (HL) and relapsed or refractory systemic anaplastic large cell lymphoma (sALCL). Brentuximab vedotin is an antibody-drug conjugate targeting the cell-membrane protein CD30 [172], which is over-expressed in HL and sALCL. The antibody-drug conjugate contains a monoclonal antibody (anti-CD30), a stable linker and the cytotoxic agent auristatin [172]. The linker has a cathepsin-cleavable peptide that upon cleavage releases auristatin. This approach seems promising, shown in clinical trials that 34 % of patients with refractory HL achieved complete remission and increased survival after treatment with brentuximab vedotin [173]. In patients with sALCL, 57 % of the patients achieved a complete remission and 29 % achieved partial remission [174].

A similar strategy is to synthesize and target a prodrug or probody to a protease with highly restricted cleavage and expression pattern such as legumain. Recently, the probody IHZ™ was developed containing a masking peptide blocking the antibody`s antigen-binding site (e.g. to the epidermal growth factor receptor). The masking peptide was connected to the antibody through a protease substrate-containing linker cleavable by uPA, matriptase, and legumain [170]. Target-mediated toxicity constitutes a major limitation for the development of therapeutic antibodies. Therefore, improved safety profile is provided with a probody in cancer. So far no clinical trials have been initiated but convincing results have also been reported for similar approaches in the construction of legumain-cleavable prodrugs based on a legumain-cleavable peptide linked to the cytotoxic drugs doxorubicin, etoposide and auristatin [37, 39, 175, 176]. In these studies cell specific targeting of the prodrugs and toxicity in legumain over-expression cells have been demonstrated both *in vitro* and *in vivo*. Recently, selective targeting of TAMs by legumain-activating prodrugs represents a novel anticancer strategy [177].

Furthermore, a DNA vaccine has been developed based on targeting legumain in TAMs [96]. The vector construct was based on the pCMV/myc/cyto vector backbone, and was incorporated with the *LGMN* gene fused to the C-terminus of a mutant polyubiquitin, which makes the antigen processing by the proteasome more efficient [178]. Mice injected with the legumain-based DNA vaccine one week prior to the injection of 2×10^5 D121 non-small lung tumor cells significantly survived longer, had reduced number of TAMs and metastasis compared to control [96].

Colchicine

Colchicine is an anti-inflammatory drug approved as monotherapy to treat inflammatory diseases such as gout and Mediterranean fever, and the use in Norway have increased by 3-fold during the last decade [179]. Recently, a clinical trial showed that low-dose use of colchicine (0.5 mg/day) had promising results also in prevention of cardiovascular disease in patients [180]. Colchicine inhibits microtubule polymerization by effectively binding to the colchicine binding site (CSB) in tubulin. CSB is one of the most important pockets for potential polymerization destabilizers such as podophyllotoxin and nocodazole [181]. Colchicine is under investigation as an anticancer drug. However, the therapeutic value of colchicine against cancer is restrained by its low therapeutic index due to its lipophilic nature. Therefore, colchicine can be exploited in a prodrug strategy, due to its cytotoxicity and lipophilic properties.

AIMS OF THE PRESENT STUDIES

The overall aim of this thesis is to expand our knowledge about the regulation of the cysteine protease legumain in human cell model systems, and exploit legumain in pharmacological interventions.

Specific aims are as follows:

- To investigate the expression, processing, activity, sub-cellular localization and secretion of legumain, as well as the interplay with cystatin E/M.
- To investigate effects of statins (atorvastatin and simvastatin) on the activity, expression and localization of legumain in macrophages and skeletal myotubes.
- To study the cytotoxic effects of a new legumain-cleavable colchicine prodrug in cells with various expression levels of active legumain.

SUMMARY OF PAPERS

Paper I

Transfected monoclonal cell lines of human embryonic kidney cells (HEK293 cells), over-expressing legumain, cystatin E/M, or both, were established and studied. The cell lines were used to investigate the regulation, localization and interplay between legumain and its most potent inhibitor, cystatin E/M. Although legumain is mainly a lysosomal enzyme and the inhibitor is a secretory protein, most interestingly the study showed that both legumain and cystatin E/M are able to be secreted in the cell medium as well as being internalized by cells. Surprisingly, cystatin E/M was present in the nucleus of cystatin E/M over-expressing HEK293 cells. These observations could explain how cystatin E/M can perform regulatory functions towards legumain both inside and outside the cell.

Paper II

Macrophages play a pivotal role in development of atherosclerosis and express lysosomal cysteine proteases such as legumain. Statins, HMG-CoA reductase inhibitors, are prescribed worldwide to patients with hypercholesterolemia. Previously, atorvastatin has been shown to down-regulate legumain mRNA in monocytes isolated from patients. In this study, the expression, activity and secretion of legumain was studied in established cellular models of monocytes/macrophages. Legumain levels were highly increased during monocyte-to-macrophage differentiation. Interestingly, most of the legumain produced in primary monocyte-derived macrophages was found to be secreted. Secretion of legumain from macrophages could explain the surprising observation of legumain present in human sera. Finally, atorvastatin treatment reduced both legumain processing, activity and secretion in macrophages (PM-derived and RAW264.7 macrophages), which could be important in understanding the pleiotropic effects of statins.

Paper III

Statins, HMG-CoA reductase inhibitors, are generally well-tolerated. However, side effects like myotoxicity occur. The mechanisms for statin-induced myotoxicity are still poorly understood. In this study, the effects of simvastatin were studied on glucose metabolism and legumain levels in human primary myotubes. A dose-dependent decrease in both glucose uptake and oxidation were observed in mature myotubes after exposure to simvastatin in concentrations not influencing cell viability. In addition, simvastatin caused a decrease in maturation and activity of legumain. Dysregulation of glucose metabolism and decreased legumain activity by simvastatin points out new knowledge about statins effects on skeletal muscle, and may contribute to the understanding of the myotoxicity observed by statins.

Paper IV

The cysteine protease legumain have remarkably restricted substrate specificity and is the only known asparaginyl endoprotease. Over-expression of legumain is reported in malignant cancers and unstable atherosclerotic plaques, and utilization of legumain is a promising approach to activate prodrugs, and thus targeting drugs. In this study we synthesized a prodrug by conjugating the legumain-cleavable peptide Suc-Ala-Ala-Asn-Val to the cytotoxic colchicine which upon cleavage by legumain released valyl colchicine.

The prodrug was shown to be more cytotoxic to cells expressing active legumain (HCT116 and M38L cells) than control cells (SW620, HEK293 and M4C cells). This indicates a relationship between toxicity of the prodrug, activity of legumain and release of valyl colchicine in the cells. Furthermore, co-administration of the prodrug with either cystatin E/M or the endocytosis inhibitor Dyngo-4a inhibited cell death, indicating that the prodrug toxicity was dependent on both asparaginyl endoprotease activity and endocytosis. This colchicine prodrug adds to a legumain-cleavable prodrug strategy approach and could possibly be of use both in targeted anti-cancer and anti-inflammatory therapy.

GENERAL DISCUSSION

Methodological considerations

Specificity of antibodies

In present work legumain, cystatin E/M, cathepsin B and L antibodies were used. Antibodies are routinely used in bioscience techniques such as immunoblotting, enzyme-linked immunosorbent assays (ELISA), immunofluorescence, immunohistochemistry (IHC), immunoprecipitation (IP) and flow cytometry. There are over 180 suppliers of antibodies worldwide offering approximately 350,000 antibodies for research purpose [182]. However, no universal standards of validation of the quality of antibodies are adapted. This causes concerns since vendors provide different levels of validation which is probably dependent on the company`s balance between making a profit and providing high quality. It is generally accepted that the responsibility to ensure proof of antibody specificity is on the purchaser/publisher and not the vendors concerning publishing. It is essential to ensure that the purchased antibody is specific, selective and reproducible for the appropriate applications to obtain reliable results.

The specificity of an antibody depends on many factors such as the type and number of immunogens as well as the antibody production method. The antibody selectivity is further complicated by the choice between monoclonal (mAbs) or polyclonal (pAbs) antibodies. The generation of mAbs and pAbs is quite different. The production of mAbs is based on single clones of hybridomas producing antibodies with high purity against a single epitope with one specific affinity. The advantages of using mAbs are that they show a high degree of specificity, but usually work in just a limited set of conditions. Polyclonal antibodies are typically produced in a suitable mammal such as rabbit or goat. An antigen is injected into the animal and the B-cells will usually produce various immunoglobulins specific for the antigen, resulting in a pool of antibodies with various affinities for the antigen. On the other hand, the advantage of pAbs is that it could be used in many different methods, but could be unselective due to several affinities towards many epitopes.

The specificity of an antibody is further determined by the type of immunogens used. The immunogens can either be a synthetic peptide or purified proteins. The advantage of synthetic peptides as immunogens is that the amino acid sequence to which the antibody binds is known. However, antibodies generated by synthetic peptides may not work well against

native proteins with intact conformation with or without post-translational modifications [183]. For example, synthetic antibodies may not be suitable for immunofluorescence, IP and IHC experiments, but could be used in analysis studying denatured proteins e.g. sodium dodecyl sulfate (SDS) immunoblotting. On the other hand, antibodies generated against purified proteins works well for proteins in their native conformation in analysis such as IP and IHC, but perhaps not for SDS immunoblotting and vice versa. The complexity of the specificity of an antibody is further complicated during fixation methods as used in e.g. immunofluorescence. The fixation process could alter the native form of a protein, thereby exposing epitopes that are not apparent in the native form and vice versa. For example, an antibody could recognize one epitope in fresh tissue, but unable to recognize the same epitope in a fixed tissue [184].

For the studies of human legumain and cystatin E/M in this thesis, well-characterized pAbs were used [40, 80, 175, 185]. The legumain (R&D systems, AF2199) and cystatin E/M (R&D systems, AF1286) pAbs were used in confocal imaging (immunofluorescence) of fixed samples and immunoblotting. Application of the pAbs in these techniques has previously been described [40, 80, 82]. The immunogens for producing these pAbs was proteins of legumain (amino acids 18-433) and cystatin E/M (amino acids 29-149), respectively. Polyclonal antibodies usually detect various forms of its target protein. For cystatin E/M both the glycosylated and unglycosylated forms are detected, and for legumain the proform, intermediate forms and active forms are detected. In the legumain ELISA experiments we used a kit (R&D Systems, BAF2199) containing a mAb for capturing and a biotinylated pAb (as described above) for detection. Well-characterized cathepsin B (Calbiochem, 219408) and L (R&D systems, AF952)) pAbs were also used in this work.

The legumain pAb showed specificity to legumain since introducing siRNA targeting legumain mRNA in cells markedly decreased the detection of legumain in cell lysates using pAbs [40]. Also, increased detection of legumain and cystatin E/M were observed in legumain and cystatin E/M over-expressing cells by the legumain and cystatin E/M pAbs, respectively (**paper I**). A murine monoclonal antibody against cystatin E/M (Santa Cruz, mouse, sc-73881) was used in confocal imaging (**paper I**) to avoid cross reactivity in overlay experiments against legumain and cystatin E/M pAbs originating from goat. The use of a lysotracker would have been useful but was difficult since the integrity of the lysosomes was affected by the fixation process. Therefore, acyl sulphatase B (a soluble lysosomal protein) mAb was used as a lysosomal marker in **paper III**.

Specificities of substrates

The legumain activity in this work was measured by recording the cleavage of the peptide substrate Z-Ala-Ala-Asn-AMC (Z-AAN-AMC, Table 1). Validation of the peptide cleavage in crude samples such as tissue homogenates and cell lysate samples is described elsewhere [27, 186]. Since legumain is the only well-characterized asparaginyl endoprotease, Z-Ala-Ala-Asn-AMC is thought to be suitable for detection of legumain activity. Also, the use of an acidic assay buffer (pH 5.8) favors the activity of cysteine proteases. The pH optimum for legumain activity is 6.4, but due to stability issues, a pH value of 5.8 is selected in this analysis [27]. The specificity of the substrate (Z-Ala-Ala-Asn-AMC) has been tested in combination with a broad cysteine cathepsin inhibitor (E-64). However, no change in cleavage of Z-Ala-Ala-Asn-AMC was observed by introducing E-64, excluding substrate cleavage by cysteine cathepsins [186]. Also, the addition of egg-white cystatin to the samples caused a complete inhibition of substrate cleavage, indicating legumain substrate specificity. Surprisingly, the cleavage of Z-Ala-Ala-Asn-AMC in THP-1 macrophages was partly inhibited by both E-64 and cystatin E/M, thus indicating the presence of an additional protease with AEP-activity in these cells (**paper II**). In **paper I**, we showed a correlation between the activity and expression of the mature form of legumain. By co-over-express legumain and cystatin E/M in M3CL cells, reduced legumain activity was observed compared to the legumain over-expressing M38L cells, indicating that the method used for legumain activity was satisfactory. Linearity of the assay was established by measuring the initial substrate cleavage rate when limiting the substrate consumption to less than 2 %. Values of K_{cat} and K_m for Z-Ala-Ala-Asn-AMC were 46 s^{-1} and 50 µM, respectively, indicating that this substrate is suitable for measuring enzyme kinetics. The ratio K_{cat}/K_M for legumain cleaving Z-Ala-Ala-Asn-AMC was estimated to be approximately $9.2*10^5$ M^{-1} S^{-1} (Table 1) [27].

Cathepsin B activity was measured in a similar way but using another peptide substrate (Z-Arg-Arg-AMC) as described in the literature [187, 188]. Z-Arg-Arg-AMC (Z-RR-AMC, Table 1) exhibits very good specificity to cathepsin B due to an Arg residue in the P2 position (Table 1). However, it should be noted that the K_{cat}/K_m ratio of cathepsin B is higher for the substrate Z-Phe-Arg-AMC than for Z-Arg-Arg-AMC. However, Z-Phe-Arg-AMC is a relative unselective substrate which can be cleaved by most papain-like cysteine endoproteases. The pH range for maximum activity of the cysteine cathepsins are between 7.5-8 [189], but due to stability issues the activity measurements are normally carried out at lower pH values (5.1-6.5; Table 1). Furthermore, dithiothreitol (DTT) should always be included in the assay due to oxidation of the active site of cysteine proteases.

Cathepsin L activity was measured by cleavage of the peptide substrate Z-Phe-Arg-AMC (Z-FR-AMC, Table 1) and the catalytic efficacy is good (Table1). Since this peptide also is cleaved by cathepsin B (see above), a negative control including a selective cathepsin B inhibitor (CA-074) was included [188].

Table 1. Substrate specificities and enzyme kinetic parameters of legumain and cysteine cathepsins.

Substrate	Enzyme	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m (M^{-1} S^{-1})	Conditions used: Buffer	pH	Temp. (°C)	Ref.
Z-AAN-AMC	Legumain	50	46	$9.2 * 10^5$	40 mM citric acid 121mM Na_2HPO_4	5.8	30	[27]
Z-RR-AMC	Cat B	390	1340	$3.4 * 10^6$	88 mM KH_2PO_4, 12 mM NA_2HPO	6.0	40	[190]
	Cat V	168	0.011	$6.5 * 10^1$	50 mM KH_2PO_4	6.5	37	[191]
	Cat K	23	0.0005	$2.2 * 10^1$	100 mM KH_2PO_4	6.5	37	[192]
	Cat S	220	0.02	$9.1 * 10^1$	50 mM NaOAc	5.5	25	[193]
Z-FR-AMC	Cat B	150	1500	$9.9 * 10^6$	88 mM KH_2PO_4, 12 mM NA_2HPO	6.0	40	[190]
	Cat L			$5.1 * 10^6$	100 mM NaOAc	5.5	37	[192]
	Cat S	20.8	2	96	90 mM $NaPO_4$	6.5	25	[194]
	Cat K	7.5	0.9	$1.2 * 10^5$	100 mM KH_2PO_4	6.5	37	[192]
	Cat V	6.4	0.71	$1.1 * 10^5$	50 mM KH_2PO_4	6.5	37	[191]
	Cat S	22	1,9	$8.6 * 10^3$	50 mM NaOAc	5.5	25	[193]
	Cat F	0.44	2.5	$5.7 * 10^6$	50 mM KH_2PO_4	6.5	25	[195]
	Cat X	63	0.22	$3.5 * 10^3$	20 mM NaOAc	5.1	37	[196]

Cell lines and primary cell cultures

In the work presented in this thesis, *in vitro* cell models of various origins have been used, i.e. commercially available cell lines (HEK293, RAW264.7, HCT116, SW620 and THP-1 cells) as well as primary cell cultures isolated from human biopsies (skeletal muscle cells) and whole blood (monocytes). Advantages of using cell lines instead of cells from primary cultures are that the cells are more easily controlled *in vitro* which make experiments easier and more reproducible. However, cells from cell lines are often dissimilar in behavior and response compared to cells *in vivo*, which makes them difficult to compare. Also, the cell lines used in our studies are immortalized cells of cancer origins except the RAW264.7 macrophages which were immortalized by transfection of a leukemia virus. Cell lines *in vitro* are able to live without the influence of physiological factors such as neuronal input, exposure of endocrine and serum components, and interplay of other cells. Although effects observed in cell lines are difficult to extrapolate to normal cells and tissues *in vivo*, *in vitro* cell models are valuable in basic research.

In this study, native HEK293 cells were transiently and stably transfected using a mixture of liposomes (Lipofectamine 2000) and either a *LGMN* pcDNA3.1 plasmid and/or a

CST6 pTracer plasmid. The HEK293 cells were used since the endogenous expressions of legumain and cystatin E/M in these cells are low, and the cells are easily transfected and cultured. The pcDNA3.1 plasmid contains a selective antibiotic resistance gene (aminoglycoside 3`-phosphotransferane, APT3`II) which upon expression makes the cells resistant to the antibiotic G418. Also, a selective antibiotic resistance gene (*Streptoalloteichus hindustanus ble* gene) was incorporated in the pTracer plasmid which upon expression makes the cells resistance to zeocin. Stably transfected monoclonal cell lines were obtained by culturing the transfected cells in the presence of either G418 and/or zeocin for the legumain (M38L), cystatin E/M over-expressing HEK293 (M4C) and double-transfected (M3CL) cells, respectively. Single cell colonies (clones) were selected and the clones with the highest protein expression of legumain (M38L) and/or cystatin E/M (M4C, M3CL) were used, respectively. Control cell clones transfected with the empty vectors did not show any difference in legumain or cystatin E/M levels compared to untransfected HEK293 cells. Therefore, native HEK293 cells were used as control cells.

The stably transfected monoclonal cell line models were mainly used in **paper I** and **IV**. Visually, the M38L cells seemed to have a faster and the M4C cells to have a slower growth rate than the native HEK293 cells. These observations were verified in a proliferation assay using the INCUCYTE™ Kinetic Imaging System. The proliferation rate of M38L cells were shown to be significantly increased compared to native HEK293 cells (Fig. 5). On the contrary, M4C cells had a significantly decreased proliferation rate compared to M38L and native HEK293 cells. These findings suggest a role of legumain and cystatin E/M in regulation of cell proliferation. Interestingly, a pro-survival role of legumain has been reported in the parasite *Blastocystis* [131]. Legumain has been shown to inactivate SET, an inhibitor of the DNAse NM23-H1 [55], and regulate the proliferation of hepatocytes [56]. Also, legumain has been shown to cleave histone 3.1 *in vitro*, suggesting a role in the DNA replication [40].

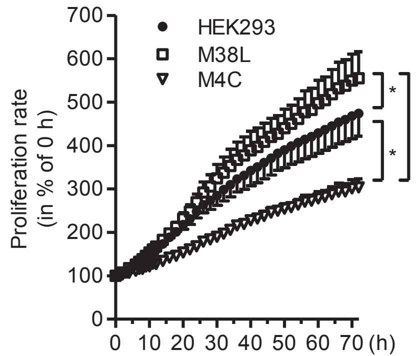

Figure 5. Proliferation of legumain over-expressing (M38L), cystatin E/M over-expressing (M4C) and native HEK293 cells. Native HEK293, M38L and M4C cells (50,000 cells/well) were cultured in 6-well plates, and the proliferation rate was measured every second hour in the **INCUCYTE™** Kinetic Imaging System (*$p<0.05$, linear regression, n=4).

In **paper II**, monocytes/macrophages were the main focus. Primary human monocytes were isolated from healthy individuals by collecting fresh full blood using Vacutainer vials with sodium heparin caps. The blood was centrifuged and the leucocytes were collected. The leucocytes were then cultured, and the monocytes were separated by adherence to the plastic well bottom. Monocytes-to-macrophages differentiation of primary monocytes was performed by stimulation with 100 ng/ml macrophage colony-stimulating factor (M-CSF), whereas the monocytes-to-macrophages differentiation of THP-1 cells was performed by incubation with 40 ng/ml PMA or 5 ng/ml TNFα.

The human primary skeletal muscle cells were derived from biopsies from healthy kidney donors at the Oslo University Hospital, and satellite cells were isolated (**paper III**). The model of *in vitro* culturing of myotubes is well-established in the laboratory, based on the method of Henry et al. 1995 and modified by Gaster et al. [197, 198]. The satellite cells are activated to proliferate and differentiate into polynuclear myotubes *in vitro* by introducing myogenic growth conditions such as the serum substitute Ultroser G, low concentration of serum (2 % FCS) and adherence to ECM. This model represents the best available *in vitro* model system for human skeletal muscle fibers (myotubes) [199]. One advantage of primary cells is that some of the innate characteristics of the donor are preserved [199]. However, work with primary cells can also be demanding, because primary cells have a limited lifespan (days) and are often subjected to infections because of the isolation process. Also, the purity

of the isolated primary satellite cells can be questionable due to frequently contamination of fibroblasts.

Subcellular localization of legumain and cystatin E/M

Legumain is suggested to have a range of cellular functions in various subcellular compartments. To proteolytically cleave its substrates, legumain must be activated at the site of substrate interaction or transported to the site in its active form. Both maturation and stability of legumain and the presence of endogenous cystatin inhibitors are factors that regulate proteolytic activity. In addition, environmental conditions and subcellular localizations are other factors that regulate the final activity of legumain. Therefore, any knowledge about the subcellular localizations and interplay of legumain and its endogenous inhibitors could potentially explain the role of legumain in different physiological or pathological settings, and could be exploited in therapeutic interventions.

The initial study on human legumain showed high activity at pH values above 6 [27], but the stability of the mature legumain was poor above pH 6. However, prolegumain is stabile in a neutral environment [27, 31], and functions of legumain in other compartments than the lysosomes have therefore been suggested [37, 40, 44]. The subcellular localizations of cysteine proteases and endogenous inhibitors seem to be altered in diseases like cancer. For example, legumain, cathepsin B and L have recently been observed in the nucleus and are suggested to participate in histone degradation [40, 109, 110, 200]. Surprisingly, we detected cystatin E/M in the nucleus of cystatin E/M over-expressing M4C cells (**paper I**). This could add to new regulation mechanisms of cysteine proteases. Previously, cystatin B and mutant cystatin C (A25T) have also been detected in the nucleus [201-203]. Although cystatin E/M is mainly a secreted protein, we showed for the first time that cystatin E/M was internalized and subsequently able to inhibit legumain activity intracellularly (**paper I**). The internalization of type 2 cystatins have previously been observed for cystatin C and F [204, 205], and has later been verified for cystatin C [206]. This clearly points out that extra- and intracellular interactions of the protease and its inhibitors are taking place, which could be of importance for the regulation of legumain activity.

Legumain is synthesized in the ER and transported as the proform to the Golgi apparatus. Thereafter, legumain is transported to the lysosomes, where legumain auto-activates at the presence of low pH. A model of the transport pathways, presumed interplay and subcellular localizations of legumain and cystatin E/M is presented below (Fig. 6). This

model shows the effects of cystatin E/M, as well as statins, on legumain trafficking and auto-activation, and will be further discussed below.

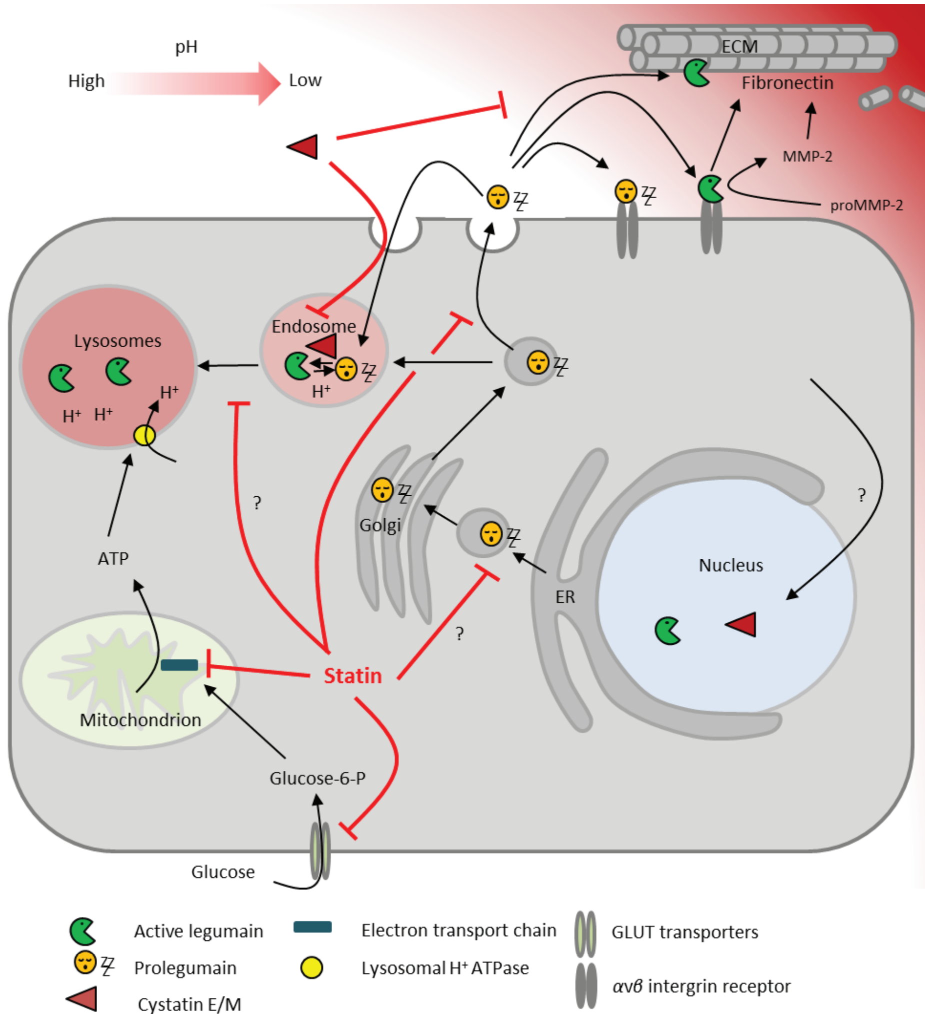

Figure 6. Model of the cellular localizations of legumain, and the effects of statins and cystatin E/M. Legumain is synthesized as an inactive proform (yellow; sleepy smiley) in the ER. Prolegumain is transported to the acidic endosomal/lysosomal compartments where it is activated (green; pac-man). Also, prolegumain can be secreted, interact with the *αvβ* integrin receptor, activated and potentially cleave extracellular substrates as e.g. fibronectin or proMMP-2. The endogenous inhibitor of legumain, cystatin E/M (red; triangle), is able to interact with active legumain both extracellularly and intracellularly. The suggested effects of statins on legumain trafficking and auto-activation, as well as glucose metabolism and ATP-production are also indicated. The pH values are scaled in a red color, and lowering of the pH values are illustrated by increasing red color.

Secretion of legumain

We observed secretion of prolegumain from over-expressing M38L cells (**paper I**), macrophages (**paper II**), human myotubes (**paper III**) and HCT116 cells (**paper IV**), respectively, suggesting that legumain also has functions as an extracellular protease. And, surprisingly, legumain (10 ng/ml) was detected in serum from healthy humans (**paper II**), indicating that legumain may be constitutively secreted. It is still poorly understood how cysteine proteases are secreted and activated in a neutral environment. However, the localization of cathepsin B, L and legumain in association with the plasma membrane and in the extracellular space have been addressed in numerous reports [106, 207-209]. Serum concentrations of cysteine proteases (cathepsin B, L and S) have been reported [210-212], and there are many examples of secreted proenzymes that become activated upon the right stimuli. For example, the serine protease thrombin in the coagulation cascade is secreted from hepatocytes as prothrombin and activated by factor Xa to the mature enzyme thrombin. It is interesting to speculate whether secreted prolegumain could be activated upon exposure to e.g. low pH, GAGs or other proteolytic enzymes. The most potent endogenous inhibitor of legumain, cystatin E/M, is mainly a secreted protein, indicating requirement for protease inhibition extracellularly. Interestingly, extracellular prolegumain seems to be stabilized at neutral pH probably due to homodimer formation [32]. Secreted prolegumain from macrophages could possibly make homodimers as indicated by gel filtration analysis (**Paper II**). Also, by increasing the pH of the lysosomes using bafilomycin A1 (a vascular H^+-ATPase inhibitor) increased secretion of prolegumain was observed (**paper I**), suggesting a switch to the exocytotic pathway versus the ER-Golgi-lysosomal pathway after neutralization of the lysosomes.

Auto-activation of prolegumain is initiated by cleavage at Asn^{323} at low pH. We showed that secreted prolegumain is auto-activated at pH 4, and that the auto-activation is inhibited by cystatin E/M due to interaction of cystatin E/M and the mature legumain (**paper I**). Recently, it has been suggested that additional cleavage at $Lys\text{-}Arg\text{-}Lys^{287}$ is required to remove the prodomain from the mature form at low pH (pH~4.5). Interestingly, the cleavage at $Lys\text{-}Arg\text{-}Lys^{287}$ can take place at pH 6 by adding trypsin, indicating that the activation of prolegumain not necessarily is auto-catalytic at low pH (pH~4.5). The auto-activation of prolegumain at slightly higher pH (pH 6) has also been observed *in vitro* in the presence of polysaccharides like GAGs and alginates [8, 35]. GAGs have been suggested to interact and pull the prodomain away resulting in exposure of the active site and subsequently legumain

activity. Since endogenous GAGs are located both extracellularly and intracellularly, they can probably facilitate both intra- and extracellular auto-activation of legumain.

Due to stability issues at neutral pH, mature legumain must be stabilized by other proteins to maintain activity in a neutral environment. Recently, association of mature legumain with the *α*v*β* integrin receptor increases the pH optimum for activity, indicating conformational stabilization of mature legumain by the *α*v*β* integrin receptor at pH values of approximately 6 [31]. Also, this can indicate extracellular localization of legumain since the *α*v*β* integrin receptor is present on the cell surface. Another opportunity to activate extracellular prolegumain can be if the pH is decreased as observed in tumor microenvironments [213], in resorptive pits between the osteoclasts and bone (also referred as extracellular lysosomes) [214], and in inflammatory acidic microenvironments. Extracellular activation of legumain may contribute to ECM degradation, progression of cancer and atherosclerosis.

Importance of legumain in macrophages

The ECM must be degraded to promote tumor progression and rupture of atherosclerotic plaques. Degradation of ECM is performed by proteases such as the MMPs and the serine protease uPA. However, cysteine proteases are also located extracellularly, and are suggested to participate in ECM degradation. For example, legumain can promote ECM degradation by activation of proMMP-2 [45], processing of cathepsin B, H and L [41], or by direct proteolysis of ECM components as shown for fibronectin (Fig. 6) [44]. Also, active forms of cathepsin B are secreted from tumor cells [106], cathepsin H from prostate tumor cells [215], and cathepsin B, K, L and S from activated macrophages [141, 216]. Prolegumain has been reported to be secreted to the acidic tumor microenvironments as well as being associated with the *α*v*β* integrin receptor at the tumor cells surfaces [37]. The cells with up-regulated legumain levels are in particular tumor cells and the tumor-associated macrophages (TAMs) [37, 96]. Recently, TAMs have gained a lot of attention since their presence is associated with poor prognosis in cancers [217-219]. TAMs are thought to be one of the key regulators of tumor progression and are targets for possible pharmacological interventions [96, 102, 177]. TAMs consist primarily of the M2 type macrophages [38, 96] and selective killing of TAMs showed reduced tumor growth, expression of angiogenic factors, number of metastasis and endothelial cells in murine tumor models [177]. Interestingly, increased levels of active

legumain and cathepsin L are also associated with macrophages within unstable atherosclerotic plaques [58, 185, 220].

Both legumain and cathepsin B were highly expressed during monocyte-to-macrophage differentiation **(paper II)**. The striking 500-fold increase in legumain mRNA expression and the 72-fold increase in activity during THP-1 cell differentiation suggest that legumain may be a potential macrophage differentiation marker (**paper II**). Legumain have recently been suggested as a M2 macrophage marker in murine models [221]. Taking into account the observed up-regulation of legumain in macrophages, measurable legumain levels in normal sera (**paper II**), as well as high legumain levels in unstable atherosclerotic plaques [58] and solid tumors [37], it is tempting to speculate that quantification of legumain could have diagnostic or prognostic value.

In **paper II**, we showed that the PMA-stimulated THP-1 macrophages polarized towards M2 macrophages, thus mimicking TAMs. Surprisingly, approximately 90 % of the total legumain produced from primary M-CSF-differentiated macrophages was secreted as proform (**paper II**). The secretion of prolegumain from macrophages, the presence of legumain in human sera (**paper II**) and subsequently internalization of secreted prolegumain (**paper I**) in neighboring or other cells suggest that prolegumain can function as an “autocrine, paracrine or endocrine factor”. The ability to distribute and regulate the amounts of extracellular proteolytic enzymes such as prolegumain can support new functions of macrophages. Furthermore, the monocytes must enter the site of the tumor or the unstable plaque by migration through the endothelial layer and basal membrane. The migration and invasion of RAW264.7 macrophages were shown to be inhibited in the presence of cystatin E/M over-expressing M4C cells compared to native HEK293 and legumain over-expressing M38L cells (Fig. 7), indicating cystatin E/M to be an inhibitor of migration and invasion of macrophages. Since M38L cells did not affect the motility or migration of RAW264.7 cells, the reduced migration of M4C cells was probably due to cystatin E/M or other proteases inhibited by cystatin E/M. However, no effects were seen on motility or invasion in similar co-culture experiments using TNFα-stimulated THP-1 cells (not shown).

In conclusion, legumain is secreted and up-regulated during monocyte-to-macrophage transition, which could contribute to ECM degradation and subsequently promotion of tumor progression and rupture of atherosclerotic plaques. Therefore, any therapeutic interventions focusing on legumain by down-regulating or utilizing legumain in a drug targeting strategy can be beneficial in e.g. cancer and atherosclerosis (see below).

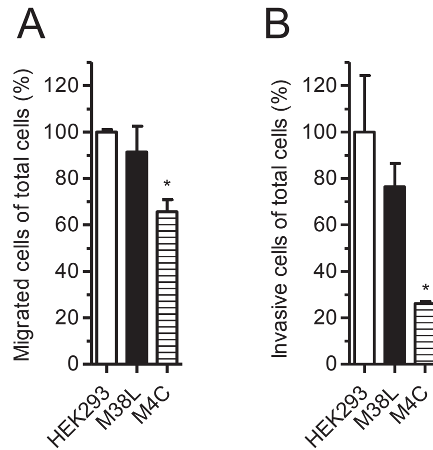

Figure 7. Motility and invasion of RAW264.7 macrophages in presence of legumain over-expressing (M38L), cystatin E/M over-expressing (M4C) and native HEK293 cells. Radiolabeled ([methyl-^{3}H] thymidine) RAW264.7 cells were cultured onto transwell permeable plate inserts with a polyester membrane coated without (**A**) or with (**B**) matrigel for 24 h, before the inserts were added to the bottom wells containing either native HEK293, M38L or M4C cells. The co-cultures were incubated for 48 hours and then harvested by collecting the RAW264.7 cells by individual swabs on each side of the polyester membranes and the radioactivity was counted in a scintillation counter. The data are normalized and calculated as radiolabeled cells below the polyester membranes divided by cells above the polyester membrane and the mean is presented (± SEM) (n=3, student t-test, *$p<0.05$).

Legumain in pharmacological interventions

Legumain inhibiton by statins

Statins (HMG-CoA reductase inhibitors) have been shown to prevent cardiovascular disease. These drugs does not only affect cholesterol synthesis but have additionally so-called pleiotropic effects, including anti-inflammatory effects involving inhibition of macrophage proliferation and reduced secretion of MMPs and MCP-1 [149]. Recently, it was demonstrated that both cathepsin K and S was down-regulated in aneurysm of the abdominal aorta after treatment with either simvastatin or atorvastatin [222, 223]. Furthermore, monocytes from patients with hypercholesterolemia treated with atorvastatin, legumain mRNA has been shown to be down-regulated [224]. However, so far no previous studies have investigated legumain expression or activity after statin exposure. Thus, in **paper II** and **III** we showed decreased protein expression, processing, activity and secretion of legumain by

two statins (atorvastatin and simvastatin, respectively) in macrophages and myotubes. Similar effects of simvastatin were observed in legumain over-expressing M38L cells, showing reduced legumain activity and processing (Fig. 8), indicating this to be a general phenomenon of statins. All cell models used in our studies responded in a similar manner to the treatment with atorvastatin or simvastatin by loss of legumain activity, although at different statin concentrations but independent of statin forms (acid or lactone form). The legumain inhibitory effects observed by statins as well as inhibition of cathepsin B activity in macrophages and myotubes (**paper II and III**) may contribute to both the beneficial and adverse effects of these drugs in patients. For example, down-regulation of legumain in macrophages could potentially add to the pleiotropic effect of statins, since legumain is suggested to participate in plaque destabilization by degradation of ECM. On the other hand, the cellular effects of statins seem to be distinct since toxic effects on skeletal muscle often is reported. Therefore, down-regulation of legumain by statins in myotubes could be unfavorable and potentially involved in statin-induced myotoxicity.

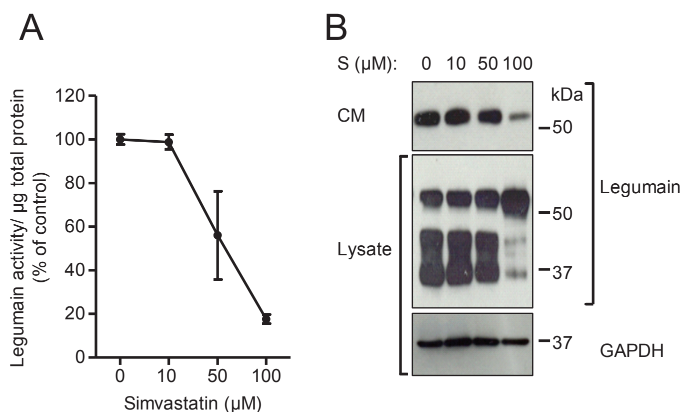

Figure 8. Inhibition of legumain by simvastatin in legumain over-expressing M38L cells. Legumain over-expressing HEK293 cells (clone M38L; 0.1 mill.) were cultured for 24 h with simvastatin (0–100 µM) before cell lysates and corresponding serum-free conditioned media were collected. **A.** Legumain activity measured in the cell lysates (±SEM) and normalized to untreated control (n=3, with duplicates). **B.** Immunoblots of equal volumes of conditioned media (upper panel) or cell lysates (12.5 µg total protein/well; middle panel) were prepared and antibody against legumain (upper and middle panels) was used. GAPDH (lower panel) was used as loading control of cell lysates.

The down-regulation of legumain activity and accumulation of the proform observed after treatment with statins could possibly be explained by several mechanisms. Since the proform auto-activates at acidic pH, it is likely that the proform is not located in the acidic lysosomes or that the lysosomes were less acidic after statin treatment. Cholesterols in the lipid bilayer regulate membrane fluidity, however, no vesicle leakage of legumain was observed (**paper III**), possibly excluding legumain translocation since activity is expected to be abolished at neutral pH [27]. Another possibility is reduced intracellular transport of newly synthesized prolegumain molecules to the lysosomes, resulting in an accumulation of the proform in intracellular vesicles with neutral pH. Interestingly, geranylgeranyl-PP has been shown to prevent decreased prenylation of Rab1 GTPase caused by fluvastatin [225], thereby inhibiting vesicular transportation from ER to Golgi. In **paper II**, both down-regulation of legumain activity and expression of the 36 kDa mature form caused by simvastatin were partly or fully prevented by geranylgeranyl-PP or mevalonolactone (ML), respectively, indicating that inhibition of prenylation of small GTPases possibly could account for some of the effects observed. Cholesterol depletion by statins could potentially lead to dysfunctional trafficking of vesicles, due to improper subcellular compartmentalization of phosphoinositides [226-228].

The accumulation of prolegumain caused by statins (**paper II and III**) was similar to the effects observed by treatment with bafilomycin A1 (**paper I**). Interestingly, re-ligation of active legumain with the C-terminal prodomain by increasing the pH has recently been reported [32], and could also explain the accumulation of the proform observed in **paper II** and **III**. The effects of legumain observed by increasing the pH led us to hypothesize that statins can increase the pH in the lysosomes.

Furthermore, glucose intolerance has been observed in tumor cells after exposure to statins [229]. Also, mitochondrial dysfunction was observed in patients taking statins [230], and was in line with the coenzyme Q10 hypothesis stating that decreased Q10 synthesis was responsible for the effects [231, 232]. Both uptake and oxidation of glucose was decreased after simvastatin treatment of the myotubes (**paper III**), and this effect could result in reduced cellular ATP, subsequently dysfunctional vacuolar type H^+–ATPase and increased pH of the lysosomes (Fig. 6). Interestingly, extracellular glucose has been reported to decrease the activity of cathepsin B, D, L and S in human monocytes and murine macrophage-like J774A.1 [233]. On the contrary, our results show enhanced legumain activity by high glucose concentrations (hyperglycemia, >5.5 mM glucose) compared to normoglycemia (5.5 mM glucose; Fig. 9). On the other hand, decreased legumain activity was observed by reducing the

glucose concentration (hypoglycemia, <5.5 mM glucose; Fig. 9). These results indicate that glucose could regulate functions of lysosomal enzymes such as legumain and could be important in diseases like diabetes. Interestingly, legumain is highly expressed in the kidney and diabetes is often associated with kidney failure [27]. It is tempting to speculate that hyperglycemia observed in diabetes could increase the activity of legumain, thus enhancing degradation of the kidney tissue and subsequently development of kidney failure.

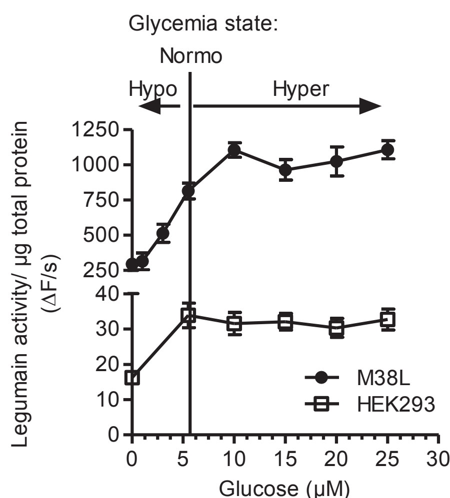

Figure 9. The glucose level regulates legumain activity in native HEK293 and legumain over-expressing M38L cells. Cells were cultured (50,000 cells/well) in 6-well plates and incubated for 6 days with different concentrations of glucose. The cells were harvested and legumain activity was measured using the substrate Z-Ala-Ala-Asn-AMC (n= 4-7).

Utilizing legumain in drug targeting

A legumain-cleavable prodrug was synthesized by conjugating colchicine to the peptide Val-Asn-Ala-Ala-Suc (Fig. 10) (**paper IV**). All of the reported prodrugs utilizing legumain have in common the legumain-cleavable peptide sequence Ala-Ala-Asn. The physiochemical properties of a drug are changed by adding a peptide sequences to a lipophilic drug such as colchicine. It becomes more hydrophilic and less cytotoxic by adding the protease-cleavable peptide connected by an amino acid linker. For example, the use of the lipophilic drug colchicine conjugated with a cathepsin B-cleavable peptide made it more hydrophilic and showed cell specific toxicity [171]. The legumain-cleavable prodrug (colchicine-Val-Asn-Ala-Ala-Suc) was more hydrophilic and less cell toxic. Thus, higher doses of the Suc-Ala-

Ala-Asn-Val-colchicine prodrug were needed to observe cell toxicity. This prodrug was cleaved by legumain, resulting in the release of the cytotoxic valyl colchicine (Fig. 10) (**paper IV**).

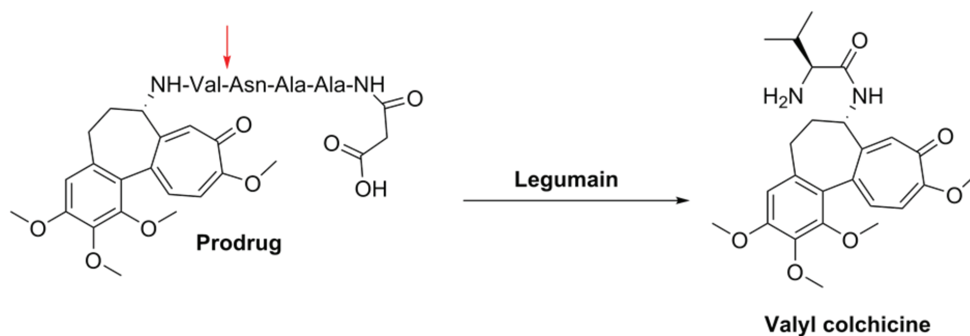

Figure 10. Synthesis of a legumain-cleavable colchicine prodrug (Suc-Ala-Ala-Asn-Val-colchicine) with cell specific toxicity. The prodrug is cleaved by legumain, resulting in release of cytotoxic valyl colchicine. The legumain cleavage site is marked by a red arrow.

It might be suggested that this prodrug could have beneficial therapeutic effects by selective prodrug cleavage in legumain over-expressing macrophages in atherosclerotic unstable plaques as well as in tumors [58, 185, 220]. The prodrug approach presented in our study could reduce the general toxicity and increase specificity of colchicine, thus extend its use against inflammation and e.g. be a possible candidate for chemotherapeutic eradication of tumors.

CONCLUSION

The novel and main findings in present work are summarized as follows:

- Secreted legumain is internalized by cells and subsequently processed and activated (**paper I**).
- Legumain activity is regulated by cystatin E/M both intra- and extracellularly (**paper I**).
- Cystatin E/M is present in the cell nucleus (**paper I**).
- Prolegumain is highly secreted from monoclonal legumain over-expressing M38L cells, macrophages, myotubes and HCT116 colorectal cancer cells (**paper I, II, III and IV**).
- The major part (90 %) of the total legumain produced in human primary M-CSF-differentiated macrophages is secreted as proform (**paper II**).
- Legumain is detectable in human sera (**paper II**).
- Legumain activity and expression are highly up-regulated during monocyte-to-macrophage differentiation, and legumain may be used as a macrophage differentiation marker (**paper II**).
- Legumain activity, expression, and secretion are inhibited by statins (HMG-CoA reductase inhibitors) in the cell types studied and may be a general phenomenon (**paper II and III**).
- Statins are able to inhibit legumain activation, probably by interference with the vesicular intracellular trafficking and/or by increasing the pH of the lysosomes. Inhibition of the HMG-CoA-reductase seems to be responsible for the reduction of legumain expression and activity induced by simvastatin (**paper III**).
- A new legumain-cleavable colchicine prodrug (Suc-Ala-Ala-Asn-Val-colchicine) is shown to be less cytotoxic than valyl colchicine or colchicine (**paper IV**).
- The legumain-cleavable colchicine prodrug has cell-specific toxicity towards cells over-expressing active legumain and could possibly be used in cell targeting (**paper IV**).

REFERENCES

1. Puente, X.S., et al., *Human and mouse proteases: a comparative genomic approach.* Nat Rev Genet, 2003. **4**(7): p. 544-58.
2. Rawlings, N.D., A.J. Barrett, and A. Bateman, *MEROPS: the database of proteolytic enzymes, their substrates and inhibitors.* Nucleic Acids Res, 2012. **40**(Database issue): p. D343-50.
3. Schechter, I. and A. Berger, *On the size of the active site in proteases. I. Papain.* Biochem Biophys Res Commun, 1967. **27**(2): p. 157-62.
4. Pungercar, J.R., et al., *Autocatalytic processing of procathepsin B is triggered by proenzyme activity.* FEBS J, 2009. **276**(3): p. 660-8.
5. Rozman, J., et al., *Autocatalytic processing of recombinant human procathepsin B is a bimolecular process.* FEBS Lett, 1999. **459**(3): p. 358-62.
6. Nishimura, Y., et al., *Evidence that aspartic proteinase is involved in the proteolytic processing event of procathepsin L in lysosomes.* Arch Biochem Biophys, 1989. **271**(2): p. 400-6.
7. Vasiljeva, O., et al., *Recombinant human procathepsin S is capable of autocatalytic processing at neutral pH in the presence of glycosaminoglycans.* FEBS Lett, 2005. **579**(5): p. 1285-90.
8. Berven, L., et al., *Autoactivation of prolegumain is accelerated by glycosaminoglycans.* Biochimie, 2013. **95**(4): p. 772-81.
9. Caglic, D., et al., *Glycosaminoglycans facilitate procathepsin B activation through disruption of propeptide-mature enzyme interactions.* J Biol Chem, 2007. **282**(45): p. 33076-85.
10. Abrahamson, M., M. Alvarez-Fernandez, and C.M. Nathanson, *Cystatins.* Biochem Soc Symp, 2003(70): p. 179-99.
11. Katunuma, N., *Mechanisms and regulation of lysosomal proteolysis.* Revis Biol Celular, 1989. **20**: p. 35-61.
12. Turk, D. and G. Guncar, *Lysosomal cysteine proteases (cathepsins): promising drug targets.* Acta Crystallogr D Biol Crystallogr, 2003. **59**(Pt 2): p. 203-13.
13. Barrett, A.J., *Human cathepsin B1. Purification and some properties of the enzyme.* Biochem J, 1973. **131**(4): p. 809-22.

14. Brix, K., et al., *Cysteine cathepsins: cellular roadmap to different functions.* Biochimie, 2008. **90**(2): p. 194-207.

15. Jordans, S., et al., *Monitoring compartment-specific substrate cleavage by cathepsins B, K, L, and S at physiological pH and redox conditions.* BMC Biochem, 2009. **10**: p. 23.

16. Roth, W., et al., *Cathepsin L deficiency as molecular defect of furless: hyperproliferation of keratinocytes and pertubation of hair follicle cycling.* FASEB J, 2000. **14**(13): p. 2075-86.

17. Halangk, W., et al., *Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis.* J Clin Invest, 2000. **106**(6): p. 773-81.

18. Li, C.Y., et al., *Mice lacking cathepsin K maintain bone remodeling but develop bone fragility despite high bone mass.* J Bone Miner Res, 2006. **21**(6): p. 865-75.

19. Riese, R.J., et al., *Regulation of CD1 function and NK1.1(+) T cell selection and maturation by cathepsin S.* Immunity, 2001. **15**(6): p. 909-19.

20. Abe, Y., et al., *Asparaginyl endopeptidase of jack bean seeds. Purification, characterization, and high utility in protein sequence analysis.* J Biol Chem, 1993. **268**(5): p. 3525-9.

21. Hara-Nishimura, I., K. Inoue, and M. Nishimura, *A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms.* FEBS Lett, 1991. **294**(1-2): p. 89-93.

22. Kembhavi, A.A., et al., *The two cysteine endopeptidases of legume seeds: purification and characterization by use of specific fluorometric assays.* Arch Biochem Biophys, 1993. **303**(2): p. 208-13.

23. Becker, C., et al., *Purification, cDNA cloning and characterization of proteinase B, an asparagine-specific endopeptidase from germinating vetch (Vicia sativa L.) seeds.* Eur J Biochem, 1995. **228**(2): p. 456-62.

24. Csoma, C. and L. Polgar, *Proteinase from germinating bean cotyledons. Evidence for involvement of a thiol group in catalysis.* Biochem. J., 1984. **222**(3): p. 769-76.

25. Hara-Nishimura, I., Y. Takeuchi, and M. Nishimura, *Molecular characterization of a vacuolar processing enzyme related to a putative cysteine proteinase of Schistosoma mansoni.* Plant. Cell, 1993. **5**(11): p. 1651-9.

26. Dalton, J.P., L. Hola-Jamriska, and P.J. Brindley, *Asparaginyl endopeptidase activity in adult Schistosoma mansoni.* Parasitology, 1995. **111 (Pt 5)**: p. 575-80.

27. Chen, J.M., et al., *Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase.* J Biol Chem, 1997. **272**(12): p. 8090-8.
28. Tanaka, T., J. Inazawa, and Y. Nakamura, *Molecular cloning of a human cDNA encoding putative cysteine protease (PRSC1) and its chromosome assignment to 14q32.1.* Cytogenet Cell Genet, 1996. **74**(1-2): p. 120-3.
29. Halfon, S., et al., *Autocatalytic activation of human legumain at aspartic acid residues.* FEBS Lett, 1998. **438**(1-2): p. 114-8.
30. Chen, J.M., et al., *Identification of the active site of legumain links it to caspases, clostripain and gingipains in a new clan of cysteine endopeptidases.* FEBS Lett, 1998. **441**(3): p. 361-5.
31. Dall, E. and H. Brandstetter, *Mechanistic and structural studies on legumain explain its zymogenicity, distinct activation pathways, and regulation.* Proc Natl Acad Sci U S A, 2013. **110**(27): p. 10940-5.
32. Zhao, L., et al., *Structural analysis of asparaginyl endopeptidase reveals the activation mechanism and a reversible intermediate maturation stage.* Cell Res, 2014.
33. Li, D.N., et al., *Multistep autoactivation of asparaginyl endopeptidase in vitro and in vivo.* J Biol Chem, 2003. **278**(40): p. 38980-90.
34. Lecaille, F., et al., *Proteinases participating in the processing and activation of prolegumain in primary cultured rat macrophages.* Biol Chem, 2004. **385**(6): p. 511-6.
35. Berven, L., et al., *Alginates induce legumain activity in RAW 264.7 cells and accelerate autoactivation of prolegumain.* Bioactive Carbohydrates and Dietary Fibre, 2013. **2**(1): p. 30-44.
36. Yamane, T., et al., *Legumain from bovine kidney: its purification, molecular cloning, immunohistochemical localization and degradation of annexin II and vitamin D-binding protein.* Biochim Biophys Acta, 2002. **1596**(1): p. 108-20.
37. Liu, C., et al., *Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy.* Cancer Res, 2003. **63**(11): p. 2957-64.
38. Wu, W., et al., *Targeting cell-impermeable prodrug activation to tumor microenvironment eradicates multiple drug-resistant neoplasms.* Cancer Res, 2006. **66**(2): p. 970-80.

39. Liu, Y., et al., *Targeting cell surface alpha(v)beta(3) integrin increases therapeutic efficacies of a legumain protease-activated auristatin prodrug.* Mol Pharm, 2012. **9**(1): p. 168-75.
40. Haugen, M.H., et al., *Nuclear legumain activity in colorectal cancer.* PLoS One, 2013. **8**(1): p. e52980.
41. Shirahama-Noda, K., et al., *Biosynthetic processing of cathepsins and lysosomal degradation are abolished in asparaginyl endopeptidase-deficient mice.* J Biol Chem, 2003. **278**(35): p. 33194-9.
42. Christensen, E.I., et al., *Membrane receptors for endocytosis in the renal proximal tubule.* Int Rev Cytol, 1998. **180**: p. 237-84.
43. Miller, G., et al., *Asparagine endopeptidase is required for normal kidney physiology and homeostasis.* FASEB J, 2011. **25**(5): p. 1606-17.
44. Morita, Y., et al., *Legumain/asparaginyl endopeptidase controls extracellular matrix remodeling through the degradation of fibronectin in mouse renal proximal tubular cells.* FEBS Lett, 2007. **581**(7): p. 1417-24.
45. Chen, J.M., et al., *Activation of progelatinase A by mammalian legumain, a recently discovered cysteine proteinase.* Biol Chem, 2001. **382**(5): p. 777-83.
46. Sepulveda, F.E., et al., *Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells.* Immunity, 2009. **31**(5): p. 737-48.
47. Manoury, B., et al., *An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation.* Nature, 1998. **396**(6712): p. 695-9.
48. Manoury, B., et al., *Asparagine endopeptidase can initiate the removal of the MHC class II invariant chain chaperone.* Immunity, 2003. **18**(4): p. 489-98.
49. Costantino, C.M., et al., *Lysosomal cysteine and aspartic proteases are heterogeneously expressed and act redundantly to initiate human invariant chain degradation.* J Immunol, 2008. **180**(5): p. 2876-85.
50. Maehr, R., et al., *Asparagine endopeptidase is not essential for class II MHC antigen presentation but is required for processing of cathepsin L in mice.* J Immunol, 2005. **174**(11): p. 7066-74.
51. Choi, S.J., et al., *Identification of human asparaginyl endopeptidase (legumain) as an inhibitor of osteoclast formation and bone resorption.* J Biol Chem, 1999. **274**(39): p. 27747-53.

52. Chan, C.B., et al., *Mice lacking asparaginyl endopeptidase develop disorders resembling hemophagocytic syndrome.* Proc Natl Acad Sci U S A, 2009. **106**(2): p. 468-73.
53. Yamane, T., et al., *Knockdown of legumain inhibits cleavage of annexin A2 in the mouse kidney.* Biochem Biophys Res Commun, 2013. **430**(2): p. 482-7.
54. Zeeuwen, P.L., et al., *Evidence that unrestricted legumain activity is involved in disturbed epidermal cornification in cystatin M/E deficient mice.* Hum Mol Genet, 2004. **13**(10): p. 1069-79.
55. Liu, Z., et al., *Neuroprotective actions of PIKE-L by inhibition of SET proteolytic degradation by asparagine endopeptidase.* Mol Cell, 2008. **29**(6): p. 665-78.
56. Andrade, V., et al., *Nucleoplasmic calcium regulates cell proliferation through legumain.* J Hepatol, 2011. **55**(3): p. 626-35.
57. Basurto-Islas, G., et al., *Activation of asparaginyl endopeptidase leads to Tau hyperphosphorylation in Alzheimer disease.* J Biol Chem, 2013. **288**(24): p. 17495-507.
58. Papaspyridonos, M., et al., *Novel candidate genes in unstable areas of human atherosclerotic plaques.* Arterioscler Thromb Vasc Biol, 2006. **26**(8): p. 1837-44.
59. Murthy, R.V., et al., *Legumain expression in relation to clinicopathologic and biological variables in colorectal cancer.* Clin Cancer Res, 2005. **11**(6): p. 2293-9.
60. Fossum, K. and J.R. Whitaker, *Ficin and papain inhibitor from chicken egg white.* Arch Biochem Biophys, 1968. **125**(1): p. 367-75.
61. Turk, B., V. Turk, and D. Turk, *Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors.* Biol Chem, 1997. **378**(3-4): p. 141-50.
62. Abrahamson, M., *Cystatins.* Methods Enzymol, 1994. **244**: p. 685-700.
63. Abrahamson, M., et al., *Isolation of six cysteine proteinase inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids.* J Biol Chem, 1986. **261**(24): p. 11282-9.
64. Alvarez-Fernandez, M., et al., *Inhibition of mammalian legumain by some cystatins is due to a novel second reactive site.* J Biol Chem, 1999. **274**(27): p. 19195-203.
65. Bode, W., et al., *The 2.0 A X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases.* EMBO J, 1988. **7**(8): p. 2593-9.

66. Abrahamson, M., et al., *Structure and expression of the human cystatin C gene.* Biochem J, 1990. **268**(2): p. 287-94.
67. Simonsen, O., A. Grubb, and H. Thysell, *The blood serum concentration of cystatin C (gamma-trace) as a measure of the glomerular filtration rate.* Scand J Clin Lab Invest, 1985. **45**(2): p. 97-101.
68. Hansen, T., et al., *Cathepsin B and its endogenous inhibitor cystatin C in rheumatoid arthritis synovium.* J Rheumatol, 2000. **27**(4): p. 859-65.
69. Lenarcic, B., et al., *Human cathepsin B and cysteine proteinase inhibitors (CPIs) in inflammatory and metabolic joint diseases.* Biol Chem Hoppe Seyler, 1988. **369 Suppl**: p. 257-61.
70. Shi, G.P., et al., *Cystatin C deficiency in human atherosclerosis and aortic aneurysms.* J Clin Invest, 1999. **104**(9): p. 1191-7.
71. Cataldo, A.M., et al., *Lysosomal proteinase antigens are prominently localized within senile plaques of Alzheimer's disease: evidence for a neuronal origin.* Brain Res, 1990. **513**(2): p. 181-92.
72. Ghiso, J., O. Jensson, and B. Frangione, *Amyloid fibrils in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of gamma-trace basic protein (cystatin C).* Proc Natl Acad Sci U S A, 1986. **83**(9): p. 2974-8.
73. Ghiso, J., B. Pons-Estel, and B. Frangione, *Hereditary cerebral amyloid angiopathy: the amyloid fibrils contain a protein which is a variant of cystatin C, an inhibitor of lysosomal cysteine proteases.* Biochem Biophys Res Commun, 1986. **136**(2): p. 548-54.
74. Levy, E., M. Jaskolski, and A. Grubb, *The role of cystatin C in cerebral amyloid angiopathy and stroke: cell biology and animal models.* Brain Pathol, 2006. **16**(1): p. 60-70.
75. Sotiropoulou, G., A. Anisowicz, and R. Sager, *Identification, cloning, and characterization of cystatin M, a novel cysteine proteinase inhibitor, down-regulated in breast cancer.* J Biol Chem, 1997. **272**(2): p. 903-10.
76. Ni, J., et al., *Cystatin E is a novel human cysteine proteinase inhibitor with structural resemblance to family 2 cystatins.* J Biol Chem, 1997. **272**(16): p. 10853-8.
77. Zeeuwen, P.L., et al., *A null mutation in the cystatin M/E gene of ichq mice causes juvenile lethality and defects in epidermal cornification.* Hum Mol Genet, 2002. **11**(23): p. 2867-75.

78. Cheng, T., et al., *Colocalization of cystatin M/E and its target proteases suggests a role in terminal differentiation of human hair follicle and nail.* J Invest Dermatol, 2009. **129**(5): p. 1232-42.
79. Vigneswaran, N., et al., *Silencing of cystatin M in metastatic oral cancer cell line MDA-686Ln by siRNA increases cysteine proteinases and legumain activities, cell proliferation and in vitro invasion.* Life Sci., 2006. **78**(8): p. 898-907.
80. Briggs, J.J., et al., *Cystatin E/M suppresses legumain activity and invasion of human melanoma.* BMC Cancer, 2010. **10**: p. 17.
81. Kioulafa, M., et al., *Methylation of cystatin M promoter is associated with unfavorable prognosis in operable breast cancer.* Int J Cancer, 2009. **125**(12): p. 2887-92.
82. Qiu, J., et al., *Invasion suppressor cystatin E/M (CST6): high-level cell type-specific expression in normal brain and epigenetic silencing in gliomas.* Lab Invest, 2008. **88**(9): p. 910-25.
83. Ai, L., et al., *Epigenetic silencing of the tumor suppressor cystatin M occurs during breast cancer progression.* Cancer Res, 2006. **66**(16): p. 7899-909.
84. Hosokawa, M., et al., *Over-expression of cysteine proteinase inhibitor cystatin 6 promotes pancreatic cancer growth.* Cancer Sci, 2008. **99**(8): p. 1626-32.
85. Ferlay, J., et al., *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.* Int J Cancer, 2010. **127**(12): p. 2893-917.
86. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
87. Mohamed, M.M. and B.F. Sloane, *Cysteine cathepsins: multifunctional enzymes in cancer.* Nat Rev Cancer, 2006. **6**(10): p. 764-75.
88. Thomssen, C., et al., *Prognostic value of the cysteine proteases cathepsins B and cathepsin L in human breast cancer.* Clin Cancer Res, 1995. **1**(7): p. 741-6.
89. Chan, A.T., et al., *Cathepsin B expression and survival in colon cancer: implications for molecular detection of neoplasia.* Cancer Epidemiol Biomarkers Prev, 2010. **19**(11): p. 2777-85.
90. Kolwijck, E., et al., *The balance between extracellular cathepsins and cystatin C is of importance for ovarian cancer.* Eur J Clin Invest, 2010. **40**(7): p. 591-9.
91. Strojnik, T., T.T. Lah, and B. Zidanik, *Immunohistochemical staining of cathepsins B, L and stefin A in human hypophysis and pituitary adenomas.* Anticancer Res, 2005. **25**(1B): p. 587-94.

92. Hirai, K., et al., *Expression of cathepsin B and cystatin C in human colorectal cancer.* Hum Pathol, 1999. **30**(6): p. 680-6.

93. Yano, M., et al., *Expression of cathepsin B and cystatin C in human breast cancer.* Surg Today, 2001. **31**(5): p. 385-9.

94. Leinonen, T., et al., *Biological and prognostic role of acid cysteine proteinase inhibitor (ACPI, cystatin A) in non-small-cell lung cancer.* J Clin Pathol, 2007. **60**(5): p. 515-9.

95. Kos, J. and T.T. Lah, *Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis and therapy in cancer (review).* Oncol Rep, 1998. **5**(6): p. 1349-61.

96. Luo, Y., et al., *Targeting tumor-associated macrophages as a novel strategy against breast cancer.* J Clin Invest, 2006. **116**(8): p. 2132-2141.

97. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.

98. Allavena, P., et al., *The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages.* Crit Rev Oncol Hematol, 2008. **66**(1): p. 1-9.

99. Wyckoff, J.B., et al., *Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors.* Cancer Res, 2007. **67**(6): p. 2649-56.

100. Osterud, B. and E. Bjorklid, *Role of monocytes in atherogenesis.* Physiol Rev, 2003. **83**(4): p. 1069-112.

101. Quatromoni, J.G. and E. Eruslanov, *Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer.* Am J Transl Res, 2012. **4**(4): p. 376-89.

102. Bingle, L., N.J. Brown, and C.E. Lewis, *The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies.* J Pathol, 2002. **196**(3): p. 254-65.

103. Lewis, C.E. and J.W. Pollard, *Distinct role of macrophages in different tumor microenvironments.* Cancer Res, 2006. **66**(2): p. 605-12.

104. Allavena, P., et al., *Anti-inflammatory properties of the novel antitumor agent yondelis (trabectedin): inhibition of macrophage differentiation and cytokine production.* Cancer Res, 2005. **65**(7): p. 2964-71.

105. Turk, V., et al., *Cysteine cathepsins: from structure, function and regulation to new frontiers.* Biochim Biophys Acta, 2012. **1824**(1): p. 68-88.

106. Roshy, S., B.F. Sloane, and K. Moin, *Pericellular cathepsin B and malignant progression.* Cancer Metastasis Rev, 2003. **22**(2-3): p. 271-86.
107. Rozhin, J., et al., *Properties of a plasma membrane-associated cathepsin B-like cysteine proteinase in metastatic B16 melanoma variants.* Cancer Res, 1987. **47**(24 Pt 1): p. 6620-8.
108. Chauhan, S.S., L.J. Goldstein, and M.M. Gottesman, *Expression of cathepsin L in human tumors.* Cancer Res, 1991. **51**(5): p. 1478-81.
109. Goulet, B., et al., *Increased expression and activity of nuclear cathepsin L in cancer cells suggests a novel mechanism of cell transformation.* Mol Cancer Res, 2007. **5**(9): p. 899-907.
110. Tedelind, S., et al., *Nuclear cysteine cathepsin variants in thyroid carcinoma cells.* Biol Chem, 2010. **391**(8): p. 923-35.
111. Buck, M.R., et al., *Degradation of extracellular-matrix proteins by human cathepsin B from normal and tumour tissues.* Biochem J, 1992. **282 (Pt 1)**: p. 273-8.
112. Ishidoh, K. and E. Kominami, *Procathepsin L degrades extracellular matrix proteins in the presence of glycosaminoglycans in vitro.* Biochem Biophys Res Commun, 1995. **217**(2): p. 624-31.
113. Mai, J., et al., *Degradation of extracellular matrix protein tenascin-C by cathepsin B: an interaction involved in the progression of gliomas.* Biol Chem, 2002. **383**(9): p. 1407-13.
114. Kobayashi, H., et al., *Effects of membrane-associated cathepsin B on the activation of receptor-bound prourokinase and subsequent invasion of reconstituted basement membranes.* Biochim Biophys Acta, 1993. **1178**(1): p. 55-62.
115. Kobayashi, H., et al., *Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (Pro-uPA).* J Biol Chem, 1991. **266**(8): p. 5147-52.
116. Gocheva, V., et al., *Distinct roles for cysteine cathepsin genes in multistage tumorigenesis.* Genes Dev, 2006. **20**(5): p. 543-56.
117. Sameni, M., K. Moin, and B.F. Sloane, *Imaging proteolysis by living human breast cancer cells.* Neoplasia, 2000. **2**(6): p. 496-504.
118. Podgorski, I., et al., *Bone microenvironment modulates expression and activity of cathepsin B in prostate cancer.* Neoplasia, 2005. **7**(3): p. 207-23.
119. Sameni, M., et al., *Functional imaging of proteolysis: stromal and inflammatory cells increase tumor proteolysis.* Mol Imaging, 2003. **2**(3): p. 159-75.

120. Chwieralski, C.E., T. Welte, and F. Buhling, *Cathepsin-regulated apoptosis.* Apoptosis, 2006. **11**(2): p. 143-9.
121. Zhao, M., U.T. Brunk, and J.W. Eaton, *Delayed oxidant-induced cell death involves activation of phospholipase A2.* FEBS Lett, 2001. **509**(3): p. 399-404.
122. Persson, H.L., et al., *Prevention of oxidant-induced cell death by lysosomotropic iron chelators.* Free Radic Biol Med, 2003. **34**(10): p. 1295-305.
123. Boya, P. and G. Kroemer, *Lysosomal membrane permeabilization in cell death.* Oncogene, 2008. **27**(50): p. 6434-51.
124. Droga-Mazovec, G., et al., *Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues.* J Biol Chem, 2008. **283**(27): p. 19140-50.
125. Cirman, T., et al., *Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins.* J Biol Chem, 2004. **279**(5): p. 3578-87.
126. Roberts, L.R., et al., *Cathepsin B contributes to bile salt-induced apoptosis of rat hepatocytes.* Gastroenterology, 1997. **113**(5): p. 1714-26.
127. Guicciardi, M.E., M. Leist, and G.J. Gores, *Lysosomes in cell death.* Oncogene, 2004. **23**(16): p. 2881-90.
128. Guicciardi, M.E., et al., *Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c.* J Clin Invest, 2000. **106**(9): p. 1127-37.
129. Schotte, P., et al., *Cathepsin B-mediated activation of the proinflammatory caspase-11.* Biochem Biophys Res Commun, 1998. **251**(1): p. 379-87.
130. Ishisaka, R., et al., *Participation of a cathepsin L-type protease in the activation of caspase-3.* Cell Struct Funct, 1999. **24**(6): p. 465-70.
131. Wu, B., et al., *Blastocystis legumain is localized on the cell surface, and specific inhibition of its activity implicates a pro-survival role for the enzyme.* J Biol Chem, 2010. **285**(3): p. 1790-8.
132. Trapani, J.A. and M.J. Smyth, *Functional significance of the perforin/granzyme cell death pathway.* Nat Rev Immunol, 2002. **2**(10): p. 735-47.
133. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.
134. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.

135. Brown, B.G., et al., *Lipid lowering and plaque regression. New insights into prevention of plaque disruption and clinical events in coronary disease.* Circulation, 1993. **87**(6): p. 1781-91.
136. Finn, A.V., et al., *Concept of vulnerable/unstable plaque.* Arterioscler Thromb Vasc Biol, 2010. **30**(7): p. 1282-92.
137. Halvorsen, B., et al., *Atherosclerotic plaque stability--what determines the fate of a plaque?* Prog Cardiovasc Dis, 2008. **51**(3): p. 183-94.
138. Shalhoub, J., et al., *Innate immunity and monocyte-macrophage activation in atherosclerosis.* J Inflamm (Lond), 2011. **8**: p. 9.
139. Huh, H.Y., et al., *Regulated expression of CD36 during monocyte-to-macrophage differentiation: potential role of CD36 in foam cell formation.* Blood, 1996. **87**(5): p. 2020-8.
140. Henney, A.M., et al., *Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization.* Proc Natl Acad Sci U S A, 1991. **88**(18): p. 8154-8.
141. Reddy, V.Y., Q.Y. Zhang, and S.J. Weiss, *Pericellular mobilization of the tissue-destructive cysteine proteinases, cathepsins B, L, and S, by human monocyte-derived macrophages.* Proc Natl Acad Sci U S A, 1995. **92**(9): p. 3849-53.
142. Strandberg, T.E., et al., *Mortality and incidence of cancer during 10-year follow-up of the Scandinavian Simvastatin Survival Study (4S).* Lancet, 2004. **364**(9436): p. 771-7.
143. Pedersen, T.R., et al., *Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). 1994.* Atheroscler Suppl, 2004. **5**(3): p. 81-7.
144. Shepherd, J., et al., *Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group.* N Engl J Med, 1995. **333**(20): p. 1301-7.
145. Illingworth, D.R. and J.A. Tobert, *HMG-CoA reductase inhibitors.* Adv Protein Chem, 2001. **56**: p. 77-114.
146. Kearney, A.S., et al., *The interconversion kinetics, equilibrium, and solubilities of the lactone and hydroxyacid forms of the HMG-CoA reductase inhibitor, CI-981.* Pharm Res, 1993. **10**(10): p. 1461-5.
147. Sakaeda, T., et al., *Effects of acid and lactone forms of eight HMG-CoA reductase inhibitors on CYP-mediated metabolism and MDR1-mediated transport.* Pharm Res, 2006. **23**(3): p. 506-12.

148. Horton, J.D., J.L. Goldstein, and M.S. Brown, *SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver.* J Clin Invest, 2002. **109**(9): p. 1125-31.

149. Liao, J.K. and U. Laufs, *Pleiotropic effects of statins.* Annu Rev Pharmacol Toxicol, 2005. **45**: p. 89-118.

150. Van Aelst, L. and C. D'Souza-Schorey, *Rho GTPases and signaling networks.* Genes Dev, 1997. **11**(18): p. 2295-322.

151. Sinzinger, H., R. Wolfram, and B.A. Peskar, *Muscular side effects of statins.* J Cardiovasc Pharmacol, 2002. **40**(2): p. 163-71.

152. Arora, R., M. Liebo, and F. Maldonado, *Statin-induced myopathy: the two faces of Janus.* J Cardiovasc Pharmacol Ther, 2006. **11**(2): p. 105-12.

153. Thompson, P.D., P. Clarkson, and R.H. Karas, *Statin-associated myopathy.* JAMA, 2003. **289**(13): p. 1681-90.

154. de Sauvage Nolting, P.R., et al., *Two-year efficacy and safety of simvastatin 80 mg in familial hypercholesterolemia (the Examination of Probands and Relatives in Statin Studies With Familial Hypercholesterolemia [ExPRESS FH]).* Am J Cardiol, 2002. **90**(2): p. 181-4.

155. Bruckert, E., et al., *Mild to moderate muscular symptoms with high-dosage statin therapy in hyperlipidemic patients--the PRIMO study.* Cardiovasc Drugs Ther, 2005. **19**(6): p. 403-14.

156. Skottheim, I.B., et al., *Statin induced myotoxicity: the lactone forms are more potent than the acid forms in human skeletal muscle cells in vitro.* Eur J Pharm Sci, 2008. **33**(4-5): p. 317-25.

157. Mutoh, T., et al., *Role of tyrosine phosphorylation of phospholipase C gamma1 in the signaling pathway of HMG-CoA reductase inhibitor-induced cell death of L6 myoblasts.* FEBS Lett, 1999. **446**(1): p. 91-4.

158. Mutoh, T., et al., *Involvement of tyrosine phosphorylation in HMG-CoA reductase inhibitor-induced cell death in L6 myoblasts.* FEBS Lett, 1999. **444**(1): p. 85-9.

159. Nishimoto, T., et al., *Comparing myotoxic effects of squalene synthase inhibitor, T-91485, and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors in human myocytes.* Biochem Pharmacol, 2003. **66**(11): p. 2133-9.

160. Sakamoto, K., et al., *Rab-small GTPases are involved in fluvastatin and pravastatin-induced vacuolation in rat skeletal myofibers.* FASEB J, 2007. **21**(14): p. 4087-94.

161. Yamazaki, H., et al., *Influence of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors on ubiquinone levels in rat skeletal muscle and heart: relationship to cytotoxicity and inhibitory activity for cholesterol synthesis in human skeletal muscle cells.* J Atheroscler Thromb, 2006. **13**(6): p. 295-307.
162. Sacher, J., et al., *Delineation of myotoxicity induced by 3-hydroxy-3-methylglutaryl CoA reductase inhibitors in human skeletal muscle cells.* J Pharmacol Exp Ther, 2005. **314**(3): p. 1032-41.
163. Kobayashi, M., et al., *Preventive effects of bicarbonate on cerivastatin-induced apoptosis.* Int J Pharm, 2007. **341**(1-2): p. 181-8.
164. Costa, A.G., et al., *Cathepsin K: its skeletal actions and role as a therapeutic target in osteoporosis.* Nat Rev Rheumatol, 2011. **7**(8): p. 447-56.
165. Drake, F.H., et al., *Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts.* J Biol Chem, 1996. **271**(21): p. 12511-6.
166. Bone, H.G., et al., *Odanacatib, a cathepsin-K inhibitor for osteoporosis: a two-year study in postmenopausal women with low bone density.* J Bone Miner Res, 2010. **25**(5): p. 937-47.
167. Riese, R.J., et al., *Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading.* Immunity, 1996. **4**(4): p. 357-66.
168. Saegusa, K., et al., *Cathepsin S inhibitor prevents autoantigen presentation and autoimmunity.* J Clin Invest, 2002. **110**(3): p. 361-9.
169. Yang, H., et al., *Cathepsin S is required for murine autoimmune myasthenia gravis pathogenesis.* J Immunol, 2005. **174**(3): p. 1729-37.
170. Desnoyers, L.R., et al., *Tumor-specific activation of an EGFR-targeting probody enhances therapeutic index.* Sci Transl Med, 2013. **5**(207): p. 207ra144.
171. Yasobu, N., et al., *Design, Synthesis, and Antitumor Activity of 4-Halocolchicines and Their Pro-drugs Activated by Cathepsin B.* Acs Medicinal Chemistry Letters, 2011. **2**(5): p. 348-352.
172. Francisco, J.A., et al., *cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity.* Blood, 2003. **102**(4): p. 1458-1465.
173. Younes, A., et al., *Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin's lymphoma.* J Clin Oncol, 2012. **30**(18): p. 2183-9.

174. Pro, B., et al., *Brentuximab vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large-cell lymphoma: results of a phase II study.* J Clin Oncol, 2012. **30**(18): p. 2190-6.

175. Stern, L., et al., *A novel antitumor prodrug platform designed to be cleaved by the endoprotease legumain.* Bioconjug Chem, 2009. **20**(3): p. 500-10.

176. Bajjuri, K.M., et al., *The legumain protease-activated auristatin prodrugs suppress tumor growth and metastasis without toxicity.* ChemMedChem, 2011. **6**(1): p. 54-9.

177. Lin, Y., et al., *Selective ablation of tumor-associated macrophages suppresses metastasis and angiogenesis.* Cancer Sci, 2013. **104**(9): p. 1217-25.

178. Ibe, S., et al., *Tumor rejection by disturbing tumor stroma cell interactions.* J Exp Med, 2001. **194**(11): p. 1549-59.

179. Norwegian Institute of Public Health, *The Norwegian Drug Wholesales Statistics*, 2014

180. Nidorf, S.M., et al., *Low-dose colchicine for secondary prevention of cardiovascular disease.* J. Am. Coll. Cardiol., 2013. **61**(4): p. 404-10.

181. Lu, Y., et al., *An Overview of Tubulin Inhibitors That Interact with the Colchicine Binding Site.* Pharmaceutical Research, 2012. **29**(11): p. 2943-2971.

182. Bordeaux, J., et al., *Antibody validation.* Biotechniques, 2010. **48**(3): p. 197-209.

183. Ramos-Vara, J.A., *Technical aspects of immunohistochemistry.* Vet Pathol, 2005. **42**(4): p. 405-26.

184. Willingham, M.C., *Conditional epitopes. is your antibody always specific?* J Histochem Cytochem, 1999. **47**(10): p. 1233-6.

185. Mattock, K.L., et al., *Legumain and cathepsin-L expression in human unstable carotid plaque.* Atherosclerosis, 2010. **208**(1): p. 83-9.

186. Johansen, H.T., C.G. Knight, and A.J. Barrett, *Colorimetric and fluorimetric microplate assays for legumain and a staining reaction for detection of the enzyme after electrophoresis.* Anal Biochem, 1999. **273**(2): p. 278-83.

187. Barrett, A.J. and H. Kirschke, *Cathepsin B, Cathepsin H, and cathepsin L.* Methods Enzymol., 1981. **80**: p. 535-61.

188. Werle, B., et al., *Assessment of cathepsin L activity by use of the inhibitor CA-074 compared to cathepsin B activity in human lung tumor tissue.* Biol Chem Hoppe Seyler, 1995. **376**(3): p. 157-64.

189. Khouri, H.E., et al., *A model to explain the pH-dependent specificity of cathepsin B-catalysed hydrolyses.* Biochem J, 1991. **275 (Pt 3)**: p. 751-7.

190. Barrett, A.J. and H. Kirschke, *Cathepsin B, Cathepsin H, and cathepsin L.* Methods Enzymol, 1981. **80 Pt C**: p. 535-61.
191. Bromme, D., et al., *Human cathepsin V functional expression, tissue distribution, electrostatic surface potential, enzymatic characterization, and chromosomal localization.* Biochemistry, 1999. **38**(8): p. 2377-85.
192. Bromme, D., et al., *Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. Functional expression of human cathepsin O2 in Spodoptera frugiperda and characterization of the enzyme.* J Biol Chem, 1996. **271**(4): p. 2126-32.
193. Bromme, D., et al., *Engineering the S2 subsite specificity of human cathepsin S to a cathepsin L- and cathepsin B-like specificity.* J Biol Chem, 1994. **269**(48): p. 30238-42.
194. Bromme, D., et al., *Functional expression of human cathepsin S in Saccharomyces cerevisiae. Purification and characterization of the recombinant enzyme.* J Biol Chem, 1993. **268**(7): p. 4832-8.
195. Wang, B., et al., *Human cathepsin F. Molecular cloning, functional expression, tissue localization, and enzymatic characterization.* J Biol Chem, 1998. **273**(48): p. 32000-8.
196. Klemencic, I., et al., *Biochemical characterization of human cathepsin X revealed that the enzyme is an exopeptidase, acting as carboxymonopeptidase or carboxydipeptidase.* Eur J Biochem, 2000. **267**(17): p. 5404-12.
197. Gaster, M., et al., *A cellular model system of differentiated human myotubes.* APMIS, 2001. **109**(11): p. 735-44.
198. Henry, R.R., et al., *Insulin action and glucose metabolism in nondiabetic control and NIDDM subjects. Comparison using human skeletal muscle cell cultures.* Diabetes, 1995. **44**(8): p. 936-46.
199. Aas, V., et al., *Are cultured human myotubes far from home?* Cell Tissue Res, 2013. **354**(3): p. 671-82.
200. Goulet, B., et al., *A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor.* Mol. Cell, 2004. **14**(2): p. 207-19.
201. Riccio, M., et al., *Nuclear localization of cystatin B, the cathepsin inhibitor implicated in myoclonus epilepsy (EPM1).* Exp. Cell. Res., 2001. **262**(2): p. 84-94.
202. Ceru, S., et al., *Stefin B interacts with histones and cathepsin L in the nucleus.* J. Biol. Chem., 2010. **285**(13): p. 10078-86.

203. Paraoan, L., et al., *Unexpected intracellular localization of the AMD-associated cystatin C variant.* Traffic, 2004. **5**(11): p. 884-95.

204. Colbert, J.D., et al., *Internalization of exogenous cystatin F supresses cysteine proteases and induces the accumulation of single-chain cathepsin L by multiple mechanisms.* J Biol Chem, 2011. **286**(49): p. 42082-90.

205. Ekstrom, U., et al., *Internalization of cystatin C in human cell lines.* FEBS J, 2008. **275**(18): p. 4571-82.

206. Wallin, H., et al., *Cystatins--Extra- and intracellular cysteine protease inhibitors: High-level secretion and uptake of cystatin C in human neuroblastoma cells.* Biochimie, 2010. **92**(11): p. 1625-34.

207. Cavallo-Medved, D. and B.F. Sloane, *Cell-surface cathepsin B: understanding its functional significance.* Curr Top Dev Biol, 2003. **54**: p. 313-41.

208. Collette, J., et al., *Biosynthesis and alternate targeting of the lysosomal cysteine protease cathepsin L.* Int Rev Cytol, 2004. **241**: p. 1-51.

209. Bailey, M.J., et al., *Extracellular proteomes of M-CSF (CSF-1) and GM-CSF-dependent macrophages.* Immunol Cell Biol, 2011. **89**(2): p. 283-93.

210. Pieper, R., et al., *The human serum proteome: Display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins.* Proteomics, 2003. **3**(7): p. 1345-1364.

211. Cox, J.M., et al., *Determination of cathepsin S abundance and activity in human plasma and implications for clinical investigation.* Analytical Biochemistry, 2012. **430**(2): p. 130-137.

212. Åkerfeldt, T. and A. Larsson, *Inflammatory response is associated with increased cathepsin B and decreased cathepsin S concentrations in the circulation.* Scandinavian Journal of Clinical and Laboratory Investigation, 2011. **71**(3): p. 203-207.

213. Gatenby, R.A. and R.J. Gillies, *Why do cancers have high aerobic glycolysis?* Nat Rev Cancer, 2004. **4**(11): p. 891-9.

214. Xia, L., et al., *Localization of rat cathepsin K in osteoclasts and resorption pits: inhibition of bone resorption and cathepsin K-activity by peptidyl vinyl sulfones.* Biol Chem, 1999. **380**(6): p. 679-87.

215. Waghray, A., et al., *Analysis of a truncated form of cathepsin H in human prostate tumor cells.* J Biol Chem, 2002. **277**(13): p. 11533-8.

216. Punturieri, A., et al., *Regulation of elastinolytic cysteine proteinase activity in normal and cathepsin K-deficient human macrophages.* J Exp Med, 2000. **192**(6): p. 789-99.

217. Nam, S.J., et al., *An increase of M2 macrophages predicts poor prognosis in diffuse large B-cell lymphoma patients treated with Rituximab-CHOP.* Leuk Lymphoma, 2014.
218. He, Y.F., et al., *High MUC2 Expression in Ovarian Cancer Is Inversely Associated with the M1/M2 Ratio of Tumor-Associated Macrophages and Patient Survival Time.* PLoS One, 2013. **8**(12): p. e79769.
219. Pantano, F., et al., *The role of macrophages polarization in predicting prognosis of radically resected gastric cancer patients.* J Cell Mol Med, 2013. **17**(11): p. 1415-21.
220. Clerin, V., et al., *Expression of the cysteine protease legumain in vascular lesions and functional implications in atherogenesis.* Atherosclerosis, 2008. **201**(1): p. 53-66.
221. Espagnolle, N., et al., *Specific Inhibition of the VEGFR-3 Tyrosine Kinase by SAR131675 Reduces Peripheral and Tumor Associated Immunosuppressive Myeloid Cells.* Cancers (Basel), 2014. **6**(1): p. 472-90.
222. Abisi, S., et al., *Effect of statins on proteolytic activity in the wall of abdominal aortic aneurysms.* Br J Surg, 2008. **95**(3): p. 333-7.
223. van der Meij, E., et al., *A clinical evaluation of statin pleiotropy: statins selectively and dose-dependently reduce vascular inflammation.* PLoS One, 2013. **8**(1): p. e53882.
224. Wang, Z.H., et al., *Pleiotropic effects of atorvastatin on monocytes in atherosclerotic patients.* Journal of Clinical Pharmacology, 2010. **50**(3): p. 311-319.
225. Sakamoto, K., I. Wada, and J. Kimura, *Inhibition of Rab1 GTPase and endoplasmic reticulum-to-Golgi trafficking underlies statin's toxicity in rat skeletal myofibers.* J Pharmacol Exp Ther, 2011. **338**(1): p. 62-9.
226. Subtil, A., et al., *Acute cholesterol depletion inhibits clathrin-coated pit budding.* Proc Natl Acad Sci U S A, 1999. **96**(12): p. 6775-80.
227. Pike, L.J. and J.M. Miller, *Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover.* J Biol Chem, 1998. **273**(35): p. 22298-304.
228. Peres, C., et al., *Modulation of phosphoinositide 3-kinase activation by cholesterol level suggests a novel positive role for lipid rafts in lysophosphatidic acid signalling.* FEBS Lett, 2003. **534**(1-3): p. 164-8.
229. Malenda, A., et al., *Statins impair glucose uptake in tumor cells.* Neoplasia, 2012. **14**(4): p. 311-23.

230. Larsen, S., et al., *Simvastatin effects on skeletal muscle: relation to decreased mitochondrial function and glucose intolerance.* J Am Coll Cardiol, 2013. **61**(1): p. 44-53.

231. Nambudiri, A.M., S. Ranganathan, and H. Rudney, *The role of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in the regulation of ubiquinone synthesis in human fibroblasts.* J Biol Chem, 1980. **255**(12): p. 5894-9.

232. Lamperti, C., et al., *Muscle coenzyme Q10 level in statin-related myopathy.* Arch Neurol, 2005. **62**(11): p. 1709-12.

233. Moheimani, F., et al., *Inhibition of lysosomal function in macrophages incubated with elevated glucose concentrations: a potential contributory factor in diabetes-associated atherosclerosis.* Atherosclerosis, 2012. **223**(1): p. 144-51.

I

II

III

Simvastatin Inhibits Glucose Metabolism and Legumain Activity in Human Myotubes

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Abstract

Simvastatin, a HMG-CoA reductase inhibitor, is prescribed worldwide to patients with hypercholesterolemia. Although simvastatin is well tolerated, side effects like myotoxicity are reported. The mechanism for statin-induced myotoxicity is still poorly understood. Reports have suggested impaired mitochondrial dysfunction as a contributor to the observed myotoxicity. In this regard, we wanted to study the effects of simvastatin on glucose metabolism and the activity of legumain, a cysteine protease. Legumain, being the only known asparaginyl endopeptidase, has caspase-like properties and is described to be involved in apoptosis. Recent evidences indicate a regulatory role of both glucose and statins on cysteine proteases in monocytes. Satellite cells were isolated from the *Musculus obliquus internus abdominis* of healthy human donors, proliferated and differentiated into polynuclear myotubes. Simvastatin with or without mevalonolactone, farnesyl pyrophosphate or geranylgeranyl pyrophosphate were introduced on day 5 of differentiation. After 48 h, cells were either harvested for immunoblotting, ELISA, cell viability assay, confocal imaging or enzyme activity analysis, or placed in a fuel handling system with [^{14}C]glucose or [^{3}H]deoxyglucose for uptake and oxidation studies. A dose-dependent decrease in both glucose uptake and oxidation were observed in mature myotubes after exposure to simvastatin in concentrations not influencing cell viability. In addition, simvastatin caused a decrease in maturation and activity of legumain. Dysregulation of glucose metabolism and decreased legumain activity by simvastatin points out new knowledge about the effects of statins on skeletal muscle, and may contribute to the understanding of the myotoxicity observed by statins.

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Introduction

Simvastatin, a HMG-CoA reductase inhibitor (statin), is prescribed worldwide to patients with hypercholesterolemia to prevent cardiovascular disease and death [1,2]. Although simvastatin is well-tolerated, side effects like myotoxicity have been reported, ranging from fatigue to life-threatening rhabdomyolysis [3,4]. Several hypotheses explaining the statin-induced myotoxicity have been put forward, but the underlying mechanism is still poorly understood. The mechanisms for statin-induced myotoxicity are probably multifactorial and at least partly due to a combination of impaired isoprenylation of trafficking proteins [5,6], altered Ca^{2+} homeostasis [7] and impaired mitochondrial respiratory function [8–10]. The mitochondrial respiratory dysfunction observed in statin-treated patients caused glucose intolerance [10]. Also, glucose intolerance was observed in tumor cells after exposure to statin, resulting in decreased glucose uptake and a higher glucose concentration in the conditioned cell medium [11]. Regarding impaired mitochondrial function, many biochemical processes are affected by impaired glucose oxidation and low ATP production, like the activity of the lysosomal H^+-ATPase, pH of the lysosomes and processing of lysosomal proteases.

Legumain (asparaginyl endopeptidase) is a cysteine protease mainly localized to the lysosomes and was first characterized in mammals in 1997 [12]. Legumain is ubiquitously expressed in mammalian tissue [12], and over-expression is associated with atherosclerotic plaque instability and cancer malignancy [13,14]. In cancer malignancy legumain has been reported to translocate from the lysosomes to the cell nucleus [15]. Legumain has also been described to participate in apoptosis of *Blastocystis* and neural cells of mice [16,17]. Furthermore, legumain contributes to the maturation process of cathepsin B and L, two other cysteine proteases [18]. Recently, down-regulation of legumain mRNA in macrophages caused by atorvastatin has been reported as well as decreased cathepsin L activity in statin-treated patients with aortic aneurysms [19,20]. Legumain cleaves peptide bonds carboxy-terminally to asparagine, as well as at aspartate residues at pH below 5 and thus acquiring caspase-like properties [13,21]. The protease is expressed as a 56 kDa proform, which is autoactivated at acidic pH to 47/46 kDa intermediate forms [22]. The intermediate legumain forms are further enzymatically processed to the mature active 36 kDa form. Also, prolegumain has been reported to be secreted as well as being associated with integrins [13], and can be internalized and subsequently autoactivated [23].

Although the biological and pathological roles of legumain are starting to be elucidated, much is still unknown. Interestingly, increasing the concentration of glucose to the media of human monocytes and murine macrophage-like J774A.1 cells are reported to down-regulate the activity of cathepsin B, D, L and S [24], thus indicating an interesting regulatory role of glucose on lysosomal enzymes.

The overall aim of this study was to investigate effects of simvastatin on glucose metabolism in human myotubes. Skeletal muscles are the major organ for glucose metabolism, and any alteration caused by simvastatin on glucose metabolism in human myotubes could shed light on mechanisms involved in adverse effects and toxicity of statins. Also, the effects of simvastatin on regulation of the cysteine protease legumain were studied in this context.

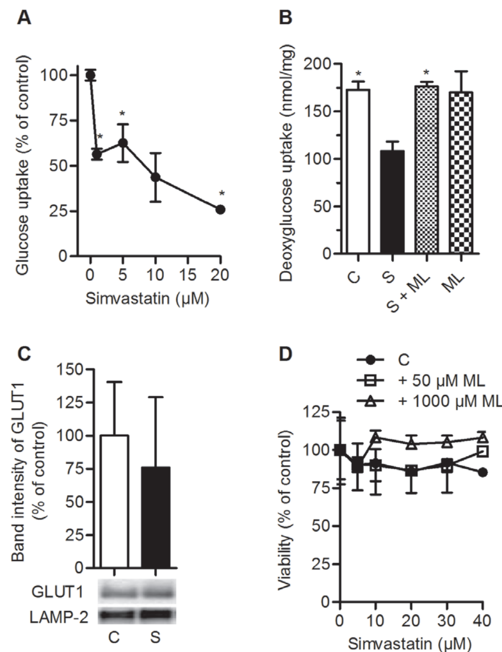

Figure 1. Reduced glucose uptake in myotubes after treatment with simvastatin. A and **B.** Differentiated myotubes were incubated for 48 h with simvastatin (S) with or without mevalonolactone (1 mM; ML) and compared to untreated control (C), prior to incubation with radiolabeled substrates at day 7. **A.** Dose-response of simvastatin on glucose uptake after 4 h incubation with [^{14}C(U)]glucose (0.2 mM, 21.5 kBq/ml) using a multiwell trapping device. Radioactivity was measured in cell lysates and in trapped CO_2 and corrected for total proteins (n = 3–8, student t-test, *p$<$0.05 vs. untreated). **B.** Effects of 5 μM simvastatin with or without ML on uptake of [^{3}H]deoxyglucose (10 μM, 37 kBq/ml, 15 min) (n = 3, student t-test, *p$<$0.05 vs. S). **C.** Differentiated myotubes were pre-incubated for 48 h with simvastatin (30 μM) before subcellular fractionation. Equal amount of total proteins from the membrane fraction were loaded to the gel and immunoblot analysis performed. LAMP-2 was used as loading control. Quantification of GLUT1 band intensity is shown, corrected for LAMP-2 and normalized to untreated control (C) (n = 4). **D.** Myotubes were treated with 0–40 μM simvastatin with or without 50 μM or 1 mM ML for 48 h before cell viability was analyzed at day 7. The cells were incubated with MTS-reagent for 2 h before absorbance at 490 nm was measured (n = 3, student t-test, *p$<$0.05 vs. untreated control (C)).
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Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM-Glutamax™, 5.5 mM glucose), foetal bovine serum, Ultroser G, penicillin–streptomycin (P/S), amphotericin B, DAPI, XCell SureLock® Mini, NOVEX Tris-Glycine Native Sample Buffer (2X), NOVEX Tris-Glycine Native Running buffer (10X), NuPAGE Bis-Tris 4–12% gels, NuPAGE MOPS SDS running buffer (20X), NuPAGE LDS sample buffer (4X), Alexa®568 donkey anti-mouse (cat. no. A10037), Alexa®488 donkey anti-goat (cat. no. A11055) and ProLong® Gold antifade reagent with DAPI (cat. no. P36935) were obtained from Life Technologies (Paisley, UK). [^{14}C-(U)]glucose (107.3 GBq/mmol), [^{3}H]deoxyglucose (37 MBq/ml) and [1-^{14}C]oleic acid (2 GBq/mmol) were purchased from PerkinElmer NEN® (Boston, MA, USA). Simvastatin was obtained from Toronto Research Chemicals (Ontario, Canada). Insulin Actrapid was from Novo Nordisk (Bagsvaerd, Denmark). Culture plates (6-, 12- and 96-wells) and 25 cm^{2} flasks were obtained from Corning Life-Sciences (Schiphol-Rijk, The Netherlands). Opti-Phase Supermix and UniFilter®-96 GF/B were delivered by PerkinElmer (Shelton, CT, USA). CHAPS, DL-dithiotreitol (DTT), trypan blue, triton X-100, mevalonolactone, geranylgeranyl pyrophosphate ammonium salt, farnesyl pyrophosphate ammonium salt, rotenone, oligomycin A, carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) and antimycin A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium pyruvate solution, foetal calf serum (FCS) and trypsin-EDTA were purchased from PAA Laboratories GmbH (Pasching, Austria). Nitrocellulose membranes were from Hybond ECL (Amersham Biosciences, Boston, MA, US). Cytoslides (cat. no. 154534), SuperSignal West Dura Extended Duration Substrate and Restore Western Blot Stripping Buffer were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Protein assay reagent, Tween 20, SDS, Precision plus protein standards, goat anti-rabbit IgG HRP-conjugate (cat. no. 170-6515) and goat anti-mouse IgG HRP-conjugate (cat. no. 170-6516) were purchased from BioRad (Copenhagen, Denmark). Goat anti-human legumain (cat. no. AF2199), goat anti-human cathepsin L (cat. no. AF952), goat anti-human cathepsin B (cat. no. AF953) and mouse anti-human arylsulfatase B (cat. no. MAB4415) were purchased from R&D Systems (Abingdon, UK). MitoProfile® Total OXPHOS Human WB Antibody Cocktail (cat. no. ab110411) and rabbit anti-human GLUT1 (cat. no. ab15309) were from Abcam (Cambridge, UK). Rabbit anti-goat IgG HRP-conjugate (cat. no. P0160) was purchased from DAKO (Glostrup, Denmark), whereas mouse anti-human α-tubulin (cat. no. CP06) was obtained from Calbiochem (San Diego, CA, USA). Mouse anti-human GAPDH (cat. no. sc-47724) and mouse anti-human LAMP-2 (sc-18822) were from Santa Cruz (Heidelberg, Germany). Z-Arg-Arg-AMC and Z-Ala-Ala-Asn-AMC were purchased from Bachem (Bubendorf, Switzerland). Non-fat dry milk was from Normilk (Levanger, Norway). Qproteome Cell Compartment Kit was purchased from Qiagen (Hilden, Germany). CellTiter 96® aqueous one solution cell proliferation assay (MTS assay) was obtained from Promega (Madison, Wisconsin, USA). All other chemicals used were standard commercial high-purity quality.

Ethics Statement

The biopsies were obtained with informed written consent and approval by the Regional Committee for Medical and Health

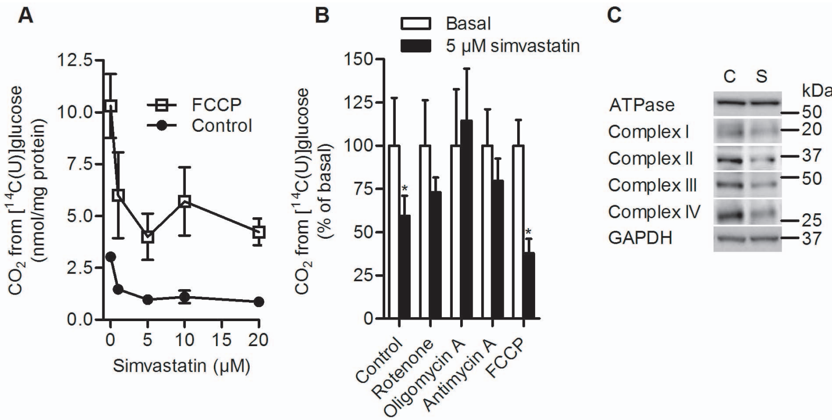

Figure 2. Effects of simvastatin on glucose oxidation and expression of proteins involved in oxidative phosphorylation. A and **B.** Differentiated myotubes were pre-incubated for 48 h with simvastatin (**A**: 0–20 µM; **B**: 5 µM) prior to incubation for 4 h with [^{14}C(U)]glucose (0.2 mM, 21.5 kBq/ml) with or without different agents at day 7. **A.** Dose-response of simvastatin on glucose oxidation with or without addition of FCCP (1 µM; n = 3). **B.** Effects on glucose oxidation of 0.1 µM rotenone, 0.1 µM antimycin A, 1 µg/ml oligomycin A or 1 µM FCCP with (black bars) or without (open bars) simvastatin. Radiolabeled [^{14}C]CO_2 was trapped and counted in a MicroBeta® scintillation counter and corrected for total protein (n = 4–8, student paired t-test, *p<0.05 vs. basal). **C.** One representative immunoblot of proteins involved in oxidative phosphorylation from differentiated myotubes incubated for 48 h with (S) or without (C) 10 µM simvastatin is shown. Twenty µg total proteins were loaded to the gel and immunoblot analysis using MitoProfile® Total OXPHOS Human WB Antibody Cocktail and GAPDH were performed (n = 3).
doi:10.1371/journal.pone.0085721.g002

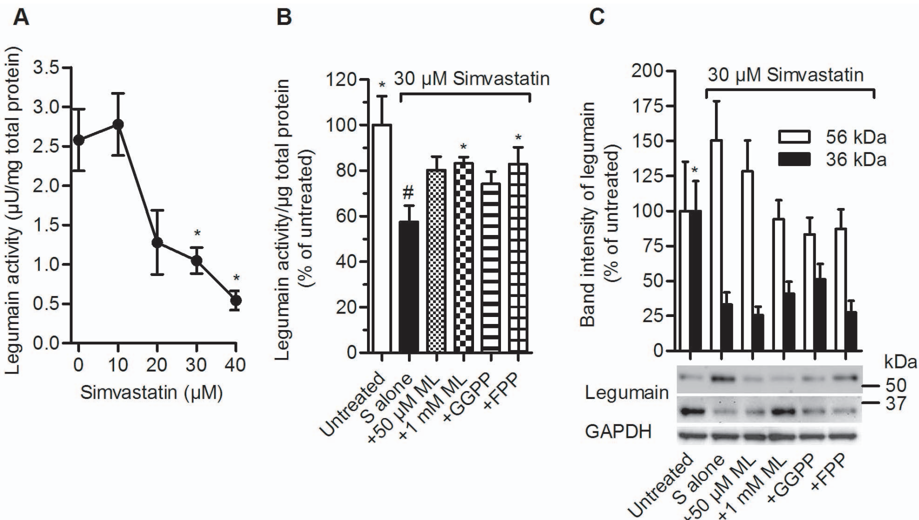

Figure 3. Legumain activity and expression after treatment with simvastatin, mevalonolactone, geranylgeranyl pyrophosphate and/or farnesyl pyrophosphate. Differentiated myotubes were incubated for 48 h with simvastatin (0–40 µM) with or without mevalonolactone (50 or 1000 µM; ML), geranylgeranyl pyrophosphate (3 µM; GGPP) or farnesyl pyrophosphate (3 µM; FPP) prior to harvesting at day 7. **A.** Dose-dependent effects of simvastatin (0–40 µM) on legumain activity (n = 3–15, student t-test, *p<0.05 vs. 0 µM). **B.** Effects on legumain activity caused by 30 µM simvastatin (S alone) with or without ML, GGPP or FPP. The data are compared and normalized to untreated myotubes (n = 6–12, student t-test, *p<0.05 vs. S alone; n = 12, paired student t-test, #p<0.01 vs. untreated). **C.** Effects on legumain expression caused by 30 µM simvastatin (S alone) with or without ML, GGPP or FPP. Equal amounts of total proteins (10 µg) of cell lysates were separated and immunoblot analyzes were performed. One representative immunoblot is shown and band intensity analysis are normalized to 36 or 56 kDa legumain immunobands, respectively, (n = 4–7, student t-test, *p<0.05 vs. S alone).
doi:10.1371/journal.pone.0085721.g003

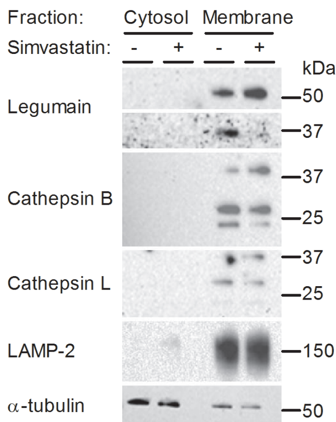

Figure 4. Legumain, cathepsin B and L expressions in cytosolic and membrane fractions of myotubes. Myotubes were treated with (+) or without (−) 30 μM simvastatin for 48 h before subcellular fractionation was performed. One representative immunoblot of legumain, cathepsin B and L in the cytosolic and membrane fractions is shown. All lanes were loaded with equal amount of total proteins and probed with antibodies as indicated. LAMP-2 and α-tubulin are shown as cell compartment controls (n = 3).
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Research Ethics (Oslo, Norway). The research performed in this study was approved, as a part of a larger project, by the Regional Committee for Medical and Health Research Ethics (Oslo, Norway).

Cell Culturing

Satellite cells were isolated from the *Musculus obliquus internus abdominis* of healthy human donors with no history of statin treatment. The cells were isolated, cultured, proliferated and differentiated as described elsewhere [25]. Briefly, cells were cultured in wells or flasks at a density of approximately 5000–30000 cells/cm^2 in medium containing DMEM-Glutamax (5.5 mM glucose), 10% FCS, 50 units/ml penicillin/streptomycin (P/S) and 1.25 μg/ml amphotericin B. This medium was substituted after one day with medium containing DMEM-Glutamax, 2% FCS, 2% Ultroser G, 50 units/ml P/S and 1.25 μg/ml amphotericin B and changed every 2–3 days until 80–90% confluence. Myoblast differentiation to myotubes was then induced by changing medium to DMEM-Glutamax with 2% FCS, 34 pM insulin, 50 units/ml P/S and 1.25 μg/ml amphotericin B. The cells were cultured, proliferated and differentiated in humidified 5% CO_2 atmosphere at 37°C. Incubation with 0–40 μM simvastatin with or without 0.05 or 1 mM mevalonolactone (ML), 3 μM farnesyl pyrophosphate (FPP) or 3 μM geranylgeranyl pyrophosphate (GGPP) in the differentiation medium were introduced on day 5 of differentiation. After 48 h of incubation, cells were either harvested in lysis buffer containing 100 mM sodium citrate, 1 mM disodium-EDTA, 1% n-octyl-β-D-glucopyranoside, pH 5.8 or used for further experiments described later. Cell lysates were freeze-thawed 3 times before analysis by immunoblotting, enzyme activity and total protein measurements. Total protein concentrations were determined by a procedure described elsewhere [26] and standard curves were established using albumin.

Cysteine Protease Activity Measurements

Legumain activity was measured by recording the cleavage of the peptide substrate Z-Ala-Ala-Asn-AMC. Briefly, 20 μl of cell lysate was added to black 96-well microtiter plates. A kinetic measurement based on increase in fluorescence over 10–60 min was performed after addition of 100 μl legumain assay buffer and 50 μl peptide substrate solution (10 μM Z-Ala-Ala-Asn-AMC) described elsewhere [12,27].

Cathepsin B activity was measured in a similar way except of using the peptide substrate Z-Arg-Arg-AMC. Briefly, 20 μl of cell lysate was added to black 96-well microtiter plates. Cathepsin B assay buffer [28,29] and peptide substrate solution (20 μM Z-Arg-Arg-AMC) was added and fluorescence measured. Temperature was kept at 30°C and all measurements were done in triplicate.

Linarites of the assays were established by measuring the initial substrate cleavage rates and limiting the substrate consumption to less than 2% during the measurements. Enzyme activity is presented as unit/mg total proteins (μmol/(min·mg)).

Immunoblotting

Samples of cell lysate were prepared for NuPAGE electrophoresis according to the manufacturer's recommendations (Life Technologies). Briefly, samples were mixed with 0.5 M DTT and NuPAGE LDS sample buffer and run along with 5 μl Precision plus protein standard on NuPAGE 4–12% gels in a container with NuPAGE MOPS SDS running buffer. Blotting was performed using 20% methanol, 25 mM Tris, and 0.2 M glycine, pH 8.3. Nitrocellulose membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1–2 h at room temperature, and then incubated overnight at 4°C with goat anti-human legumain (1:1000), goat anti-human cathepsin B (1:10000), goat anti-human cathepsin L (1:5000), mouse anti-human GAPDH (1:10000), mouse anti-human LAMP-2 (1:500), mouse anti-human α-tubulin (1:5000), rabbit anti-human GLUT1 (1:1000), mouse anti-human total OXPHOS cocktail (1:500) or mouse anti-human arylsulfatase B (ARSB; 1:500). Further incubation for one hour was performed with appropriate HRP-conjugate of secondary antibodies. After four ten-minute washes in TBS-T, immunoreactive bands on the membranes were detected by SuperSignal West Dura Extended Duration Substrate. Membranes were reprobed after stripping in Restore Western Blot Stripping Buffer as described by the manufacturer (Thermo Fisher Scientific). Immunoband intensities were analyzed by Image 4.0 (BioRad).

Confocal Imaging

Cells (5×10^4) were seeded on cytoslides, cultured, differentiated and treated as described above. On day 7 after start of differentiation, cells were fixed with 4% paraformaldehyde in PBS for 10 min on ice and washed twice in PBS before being permeabilized for 5 min with 0.2% Triton X-100. Cells were washed three times with PBS, 0.1% BSA, 0.2% Triton X-100 and 0.05% Tween 20, and then blocked with 10% horse serum for 1 h. Then, immunocytochemical staining was performed using goat anti-human legumain (1:50) or goat anti-human cathepsin B (1:100) and mouse anti-human ARSB (1:50) primary antibodies for 1 h. After three washes, corresponding secondary antibodies were applied (donkey anti-goat; Alexa 488; 1:250 or donkey anti-mouse; Alexa 568; 1:500, respectively). The coverslips were mounted in ProLong Gold antifade reagent with DAPI. The cells were observed using a laser-scanning confocal imaging system LSM710 (Carl Zeiss) with equal settings in all experiments.

ELISA

Established ELISA procedure given by the manufacturer was performed to measure the concentrations of legumain (R&D Systems; MAB21992) in conditioned media from myotubes.

Cell Viability (MTS)

Cell viability assays were carried out using the manufacturer's protocol. Briefly, 5×10^4 cells were cultured, proliferated, differentiated and treated with simvastatin in quadruplicates in 96-wells culture plates. After 24 h, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS reagent) were added to each well and incubated for 2 h before absorbance was measured at 490 nm in a microplate reader, Wallac Victor^3 (PerkinElmer).

Subcellular Fractionation

Samples of different subcellular fractions were prepared using the Qproteome Cell Compartment Kit according to the manufacturer's protocol. Purity of fractions was checked with α-tubulin, LAMP-2, ARSB and Lamin B as cytosolic, membrane, soluble membrane and nuclear markers, respectively.

Uptake and Oxidation of Glucose

Cells were cultured, proliferated and differentiated on 96-well CellBIND® microplates. First, 48 h incubation with simvastatin was started on day 5 of differentiation before analysis with cell-based multiwell assay was performed as described elsewhere [30]. Briefly, medium was removed before addition of [^{14}C-(U)]glucose (37 kBq/ml, 0.2 mM) in Dulbeccòs PBS (DPBS) with 10 mM HEPES, containing either 0.1% DMSO, 0.1 µM rotenone, 1 µg/ml oligomycin A, 0.1 µM antimycin A, or 1 µM FCCP. A 96-well UNIFILTER® microplate presoaked with 20 µl 1 M NaOH was mounted on top of the CellBIND® plate, and the cells were incubated at 37°C and 5% CO_2 for 4 h. The CO_2 trapped in the filter was then counted by liquid scintillation in a MicroBeta™ Trilux scintillation counter (PerkinElmer). The remaining cell-associated radioactivity was also assessed by liquid scintillation, and the formation of CO_2 and cell-associated radioactivity was considered as total glucose uptake while the formation of CO_2 was considered as glucose oxidation.

Uptake of Deoxyglucose

The cells were cultured, proliferated and differentiated on 12-well plates. First, 5 days after onset of differentiation, treatment with 5 µM simvastatin with or without 1 mM ML were added. After 48 h of incubation, the cells were washed and incubated for 1 h with 140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM $MgSO_4$ and 1 mM $CaCl_2$, pH 7.4, before [^{3}H]deoxyglucose (37 kBq/ml, 10 µM) was added and incubated for 15 min. Then cells were washed 3 times with PBS and harvested in 250 µl 0.1 M NaOH. The lysates were counted by liquid scintillation.

Statistics

The data are represented as mean ± SEM. Student t-test or student paired t-test were performed when appropriate, and statistical significance was considered at $p<0.05$. All experiments were performed on cells from at least three donors and at least triplicate measurements.

Results

Simvastatin Reduced Glucose Uptake and Oxidation in Human Myotubes

Previous reports have shown that simvastatin reduces glucose metabolism both in cancer cells and adipocytes [11,31]. In our study using differentiated human myotubes, simvastatin significantly reduced uptake of [^{14}C]glucose in a dose-dependent manner with an IC_{50} value of approximately 8 µM (Fig. 1A). The myotubes seemed to be more sensitive to reduced glucose uptake by simvastatin than the embryonic kidney cell line HEK293. Whereas 5 µM simvastatin significantly decreased glucose uptake in the myotubes, no effect was observed in the HEK293 cells (Fig. S1). Reduced glucose uptake caused by 5 µM simvastatin was confirmed using [^{3}H]deoxyglucose giving approximately 45% less uptake (Fig. 1B). Treatment with a combination of simvastatin and mevalonolactone (ML; 1 mM) totally prevented the decreased deoxyglucose uptake (Fig. 1B). Since the experiments were done in absence of insulin and GLUT1 is the predominant glucose transporter in human myotubes [32], the GLUT1 expression after exposure to simvastatin was studied. The observed effects of simvastatin were not due to differences in GLUT1 expression (Fig. 1C). To verify that the observed effects of simvastatin were not due to cell death of myotubes, cell viability, total proteins concentrations and caspase-3 expression were studied. There were no differences in cell viability (Fig. 1D), total protein concentrations (Fig. S2A) or immunoband of active caspase-3 (Fig. S2B) after simvastatin treatment.

Knowing that the myotubes were fully viable at the simvastatin concentrations used, the observed reduction in glucose uptake could be an indirect effect of impaired oxidation of glucose. Therefore, an uncoupler of oxidative phosphorylation (FCCP) was introduced and shown to increase oxidation of glucose by approximately 3-fold. This reflected a high reserve capacity for glucose oxidation in myotubes, calculated as the difference between oxidation in the presence or absence of FCCP (Fig. 2A). The reserve capacity for glucose oxidation after simvastatin (5 µM) treatment was reduced by approximately 40% (Fig. 2A). To further study the mechanism of simvastatin (5 µM) on oxidative phosphorylation, different inhibitors of the respiratory chain and ATP formation were introduced. Rotenone, oligomycin A, and antimycin A were used to inhibit complex I, ATP synthase and complex III, respectively. No significant effects on glucose oxidation were observed in myotubes treated with any of these agents with or without simvastatin, whereas treatment with FCCP reflected the observation already described above (Fig. 2B). Finally, the effects of simvastatin on the expression of complex I, complex II subunit 30 kDa, complex III core 2, complex IV and ATPase-α-subunit were studied. No significant differences were observed, but there was a tendency of reduced expression of complex I-IV of the respiratory chain by simvastatin (Fig. 2C; Fig. S3).

Reduced Legumain Activity and Expression in Simvastatin-treated Myotubes

The glucose concentration in the cell culture media has been reported to regulate the activity of cathepsin B, D, L and S in human monocytes and murine macrophage-like J774A.1 cells [24]. Also, atorvastatin has been shown to decrease legumain mRNA in monocytes [20]. Since glucose metabolism was decreased in human myotubes after exposure to simvastatin (Fig. 1, 2), this interesting regulatory role of glucose on lysosomal enzymes led us to investigate whether legumain was affected by simvastatin in human myotubes. Initially, expression and activity

of legumain were studied during myotube differentiation and compared to cathepsin B, which is reported to participate in myotube differentiation [33,34]. Undifferentiated myoblasts (differentiation day 0) showed both legumain and cathepsin B activity at a level of 0.9 (±0.2) and 5.8 (±1.1) μUnit/mg, respectively. Legumain activity was significantly increased both at differentiation day 2 and 5 compared to day 0 (Fig. S4A). Increased activity was due to increased expression of the mature active form (36 kDa) as reflected by immunoblotting (Fig. S4B and C). Cathepsin B activity and expression of the active two-chain form (23 kDa) also showed increasing tendencies throughout myotube differentiation (Fig. S4A, B and D).

Treatment of myotubes on day 5 of differentiation with increasing concentrations of simvastatin for 48 h showed reduced legumain activity in a dose-dependent manner with an IC_{50} value of about 25 μM (Fig. 3A). There was also a tendency of reduced cathepsin B activity caused by increasing simvastatin concentration (data not shown). To study whether inhibition of the HMG-CoA reductase was involved in the reduced legumain activity observed by simvastatin, intermediates of the mevalonate pathway including mevalonolactone (ML), geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were introduced. The effect of simvastatin (30 μM) on legumain activity was partly prevented by ML (1 mM) or FPP (3 μM; Fig. 3B). Also, the legumain activity measurement was reflected by the expression of the 36 kDa immunoband, representing the mature active form, which was significantly reduced after treatment with simvastatin alone (Fig. 3C, S alone). In addition, a concomitant accumulation of the 56 kDa immunoband was seen, reflecting reduced prolegumain processing. No significant changes were observed by addition of ML, GGPP or FPP (Fig. 3C). Furthermore, fully differentiated myotubes secreted legumain to the conditioned media at a rate of 0.02 (±0.005) pg/cell/day, and the secretion was not affected by treatment with simvastatin (30 μM).

To study whether simvastatin could alter the intracellular distribution of lysosomal cysteine proteases, subcellular compartments of myotubes were isolated and analyzed for legumain, cathepsin B and L. Immunoblots showed that both legumain, cathepsin B and L in untreated myotubes were located only in the membrane compartment, comprising cytoplasmic organelles (Fig. 4). After simvastatin treatment, there was no altered subcellular localization of either legumain, cathepsin B or L, as no immunbands were detected in the cytosolic (Fig. 4) or nuclear compartments (data not shown). Subcellular compartment purities were verified by LAMP-2 (membranes), α-tubulin (cytosol) and lamin B (nucleus; not shown), respectively. Simvastatin (30 μM) reduced the expression level of the mature form of legumain (36 kDa) as well as increased the level of prolegumain (56 kDa) in the membrane fraction, confirming the observation in whole myotube lysates (Fig. 3). Also, subcellular presence of legumain and cathepsin B was confirmed by confocal imaging. Legumain seemed to be co-localized in lysosomes with arylsulfatase B (ARSB, a soluble lysosomal enzyme) in untreated myotubes (yellow; Fig. S5A) and treatment with simvastatin did not seem to alter legumain distribution (Fig. S5B). Cathepsin B also seemed to be vesicular although not distinctly co-localized with ARSB (Fig. S5C), and simvastatin caused a more diffuse staining (Fig. S5D).

Discussion

In this study we observed decreased uptake and oxidation of glucose in human myotubes caused by treatment with simvastatin. Also, legumain (a cysteine protease) was for the first time characterized in human myotubes and decreased legumain activity and expression was observed by simvastatin. The reduction in legumain activity was caused by decreased processing of the 56 kDa prolegumain due to inhibition of the HMG-CoA reductase by simvastatin. These effects of simvastatin on differentiated human myotubes may contribute to the understanding of the pharmacology and toxicology of statins.

The glucose metabolism in human myotubes decreased upon simvastatin treatment, and it is tempting to speculate if this could contribute to hyperglycemia, since some patients taking statins develop decreased insulin sensitivity, insulin resistance and glucose intolerance [10,35,36]. Furthermore, the observed decrease in glucose uptake was prevented by concomitant addition of mevalonolactone, suggesting that the mechanism was due to direct inhibition of the HMG-CoA reductase. Although no difference in expression of GLUT1 was observed after simvastatin treatment in this study, regulation of activity or translocation of the GLUT1 transporter or other GLUT transporters could nevertheless account for the effects observed but was not investigated further. Previously, simvastatin has been reported to impair complex I and II in the respiratory chain resulting in ROS accumulation in primary human myotubes established from satellite cells isolated from another source *(Musculus vastus lateralis)* [9]. In our study we detected no differences in CO_2-production by simvastatin in presence of rotenone (complex I inhibitor), oligomycin A (ATP-synthase inhibitor) or antimycin A (complex III inhibitor). Deviant observations could be due to differences in both origin of myotubes and methods used [9]. Surprisingly, simvastatin reduced FCCP-induced glucose oxidation and oxidative reserve capacity, indicating some effects of simvastatin on cell respiration and mitochondrial function. Therefore, the expressions of proteins in the respiratory chain (complex I-IV and ATP synthase) were studied after treatment with simvastatin. Although no statistically significant changes in the expressions of the analyzed complexes were observed, the expressions tended to decrease and could account for some of the effects.

There may be a link between the two effects observed of simvastatin, impaired glucose metabolism and prolegumain processing, as reduced supply of glucose could cause ATP-depletion in the cell. Decreased ATP levels due to mitochondrial dysfunction have previously been reported in skeletal muscle cells from patients with type 2 diabetes and in rat L6 GLUT4myc myotubes acquiring impaired glucose metabolism [37,38]. We have recently shown that bafilomycin A1 (a strong inhibitor of the vacuolar type H^+-ATPase) also reduced the activity of legumain [23]. Since the lysosomal H^+-ATPase needs ATP to accomplish acidic lysosomal pH [39], our results may indicate that simvastatin could reduce the H^+-ATPase activity due to possible ATP-depletion resulting in increased lysosomal pH and thus reduced prolegumain processing.

Extracellular glucose is reported to regulate the activity of the cysteine proteases cathepsin B, D, L and S in human monocytes and murine macrophage-like J774A.1 cells [24]. This, together with our observations that simvastatin decreased glucose metabolism in myotubes and the reported down-regulation of legumain mRNA by atorvastatin observed in monocytes [20], made us study the regulatory effects of simvastatin on legumain. We observed a reduced activity of legumain caused by simvastatin and speculated if this could be due to a translocation from the lysosomes to the cytosol since such translocation of legumain has been reported in apoptosis [17]. As a HMG-CoA reductase inhibitor, simvastatin blocks the rate limiting step in the cholesterol synthesis. Cholesterol is a major component of lipid bilayers and cholesterol removal from

lysosomal membranes increases the permeability of especially ions and protons, resulting in osmotic imbalance, destabilization and potential leakage of proteins [40,41]. No lysosomal translocation of legumain was observed using 30 μM simvastatin and could thus not explain the reduced legumain activity observed since the activity is expected to be abolished at neutral cytosolic pH [12]. Cholesterol depletion has also been reported to lead to improper subcellular compartmentalization of phosphoinositides, which are essential for trafficking of intracellular vesicles [42–44]. The delocalization of phosphatinositoles is partly due to regulation by cholesterol of the activity of various phosphatinositol kinases at distinct intracellular compartments e.g. type II phosphatidylinositol 4-kinase IIα at the Golgi membrane (PI4KIIα) [45,46]. Prolegumain needs acidic pH to autoactivate [22]. Therefore, it is possible that some of the effect of simvastatin on inhibition of legumain activity could be due to dysfunctional transport of prolegumain from the Golgi to vesicles with acidic pH, like the late endosomes/lysosomes. Also, depletion of isoprenoids, mainly the geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), have been suggested to be critical for statin-induced myopathy due to their role in prenylation of small GTPases [5,6,47]. Inhibition of prenylation will lead to improper intracellular trafficking since functionally small GTPases such as Ras and Rab are essential for targeting, tethering, uncoating and formation of intracellular vesicles [5,47,48]. GGPP has been shown to prevent the decreased prenylation of Rab1 GTPase by fluvastatin [6]. Here, down-regulation of legumain activity by simvastatin was significantly prevented by FPP or mevalonolactone (ML), but not by GGPP, indicating that inhibition of prenylation of Rab1 GTPase may not account for the effects observed.

The activity of cysteine proteases are strictly controlled within a cell, and uncontrolled legumain activity is associated with serious diseases like atherosclerotic plaque instability and malignant cancer [13,14]. A pro-survival role of legumain has been reported in the parasite *Blastocystis* of which inhibition of legumain activity is associated with increased programmed cell death [16]. In this study legumain activity and expression were decreased by simvastatin, but not cell viability, cell total protein content or caspase-3 expression indicating that proteases other than legumain are involved in statin-induced cell death. Down-regulation of legumain could still contribute to a general distortion of protease/kinase activity possibly leading to toxicity, but this needs to be further investigated.

Simvastatin was introduced 5 days into myotube differentiation to mimic an *in vivo* condition since reports have demonstrated that toxic effects of statins mainly affect differentiated myotubes and not myoblasts [6,9]. Simvastatin was used in our study since this statin has the highest frequency of reported myotoxic effects [49], and the lactone forms of statins are more potent in causing myotoxicity [50]. The concentration of simvastatin used in present study is in the micromolar range, but the achieved plasma concentration after administration of a clinical simvastatin dose is in the nanomolar range. Still, the statin concentrations used in this study are in range with other published *in vitro* experiments [50]. Since the exposure of simvastatin *in vitro* must be for shorter periods of time (days) compared to long term therapeutic *in vivo* use, and the primary cell culture has limited life span, it can be argued that higher statin concentrations are needed to detect cellular responses. Nevertheless, in our study and as mentioned above, the simvastatin concentrations used in this study (5–40 μM) caused no significantly effects on cell viability, cell total protein contents or expression of active caspase-3. In contrast, it has previously been reported reduced myotube viability by 5 μM simvastatin [9,51].

In conclusion, this study shows that simvastatin reduced both glucose metabolism and legumain activity in myotubes. Both phenomenon are of importance to fully understand the pharmacology and toxicology of statin treatment and needs to be further investigated.

Supporting Information

Figure S1 Simvastatin reduced glucose uptake in myotubes but not in HEK293 cells. Differentiated myotubes or HEK293 cells were incubated for 48 h with or without 5 μM simvastatin prior to incubation for 4 h with [^{14}C(U)]glucose (0.2 mM, 21.5 kBq/ml) using a multiwell trapping device. Radioactivity was measured in cell lysates and corrected for total proteins (n = 4–8, student t-test, *$p<0.05$ vs. untreated).
(TIF)

Figure S2 Effects of simvastatin on total protein content (A) and caspase-3 expression (B) in differentiated myotubes. Differentiated myotubes were incubated for 48 h with or without simvastatin (5–40 μM). **A.** Total protein concentrations in cell lysates were measured and normalized to untreated control (n = 17). **B.** Equal amounts of total proteins (10 μg) of cell lysates were analyzed for caspase-3 by immunoblotting. One representative immunoblot is shown and band intensity analysis of procaspase-3 are normalized to untreated control (n = 3).
(TIF)

Figure S3 Expressions of ATPase, complex I, II, III and IV after simvastatin treatment. Differentiated myotubes were pre-incubated for 48 h with or without (control) simvastatin (10 or 30 μM; pooled results) prior to harvesting at day 7. Ten-twenty μg total proteins were loaded per well and immunoblotting using MitoProfile® Total OXPHOS Human WB Antibody Cocktail was performed. GAPDH was used as loading control. Quantification of immunobands are shown, corrected for GAPDH and normalized to control (n = 7).
(TIF)

Figure S4 Characterization of legumain and cathepsin B during differentiation of human myotubes. Myoblasts (50,000 cells/well) were cultured and proliferated to 80–90% confluence before start of differentiation (day 0), and cells were harvested at day 0, 2, 5 and 7. **A**. Legumain and cathepsin B activities in cell lysates were measured by cleavage of fluorogenic peptide substrates (n = 3–4, student t-test, *$p<0.05$ vs. differentiation day 0). **B.** Representative immunoblots of cell lysates are shown. Equal amounts of total proteins (10 μg/well) were applied to the gel and immunoblot analysis was performed. Immunoband intensities of pro- and active forms of legumain (**C**) and cathepsin B (**D**) were measured and normalized to differentiation day 0 (n = 3).
(TIF)

Figure S5 Localization of legumain and cathepsin B in myotubes treated with or without simvastatin. Myotubes were cultured, differentiated, and incubated for 48 h without (control) or with 30 μM simvastatin prior to fixation at day 7. After fixation, the cells were permeabilized, blocked and further incubated with primary antibodies against legumain (green; A and B) or cathepsin B (green; C and D) and arylsulfatase B (ARSB; red). Secondary antibodies against the species of the primary antibodies and DAPI (blue) were used. The cells were photo-

graphed with identical camera and laser settings by LSM710 confocal microscopy (scale bars, 20 μm).
(TIF)

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Author Contributions

Conceived and designed the experiments: R. Smith R. Solberg ACR GHT HTJ. Performed the experiments: R. Smith LLJ ALV. Analyzed the data: R. Smith LLJ ALV. Contributed reagents/materials/analysis tools: R. Smith R. Solberg ACR GHT HTJ. Wrote the paper: R. Smith. Read and approved the paper: R. Smith R. Solberg LLJ ALV ACR GHT HTJ.

References

1. Strandberg TE, Pyorala K, Cook TJ, Wilhelmsen L, Faergeman O, et al. (2004) Mortality and incidence of cancer during 10-year follow-up of the Scandinavian Simvastatin Survival Study (4S). Lancet 364: 771–777.
2. Pedersen TR (1994) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet 344: 1383–1389.
3. Sinzinger H, Wolfram R, Peskar BA (2002) Muscular side effects of statins. J Cardiovasc Pharmacol 40: 163–171.
4. Arora R, Liebo M, Maldonado F (2006) Statin-induced myopathy: the two faces of Janus. J Cardiovasc Pharmacol Ther 11: 105–112.
5. Sakamoto K, Honda T, Yokoya S, Waguri S, Kimura J (2007) Rab-small GTPases are involved in fluvastatin and pravastatin-induced vacuolation in rat skeletal myofibers. FASEB J 21: 4087–4094.
6. Sakamoto K, Wada I, Kimura J (2011) Inhibition of Rab1 GTPase and endoplasmic reticulum-to-Golgi trafficking underlies statin's toxicity in rat skeletal myofibers. J Pharmacol Exp Ther 338: 62–69.
7. Sirvent P, Mercier J, Vassort G, Lacampagne A (2005) Simvastatin triggers mitochondria-induced Ca2+ signaling alteration in skeletal muscle. Biochem Biophys Res Commun 329: 1067–1075.
8. Ghirlanda G, Oradei A, Manto A, Lippa S, Uccioli L, et al. (1993) Evidence of plasma CoQ10-lowering effect by HMG-CoA reductase inhibitors: a double-blind, placebo-controlled study. J Clin Pharmacol 33: 226–229.
9. Kwak HB, Thalacker-Mercer A, Anderson EJ, Lin CT, Kane DA, et al. (2012) Simvastatin impairs ADP-stimulated respiration and increases mitochondrial oxidative stress in primary human skeletal myotubes. Free Radic Biol Med 52: 198–207.
10. Larsen S, Stride N, Hey-Mogensen M, Hansen CN, Bang LE, et al. (2013) Simvastatin effects on skeletal muscle: relation to decreased mitochondrial function and glucose intolerance. J Am Coll Cardiol 61: 44–53.
11. Malenda A, Skrobanska A, Issat T, Winiarska M, Bil J, et al. (2012) Statins impair glucose uptake in tumor cells. Neoplasia 14: 311–323.
12. Chen JM, Dando PM, Rawlings ND, Brown MA, Young NE, et al. (1997) Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. J Biol Chem 272: 8090–8098.
13. Liu C, Sun C, Huang H, Janda K, Edgington T (2003) Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. Cancer Res 63: 2957–2964.
14. Papaspyridonos M, Smith A, Burnand KG, Taylor P, Padayachee S, et al. (2006) Novel candidate genes in unstable areas of human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 26: 1837–1844.
15. Haugen MH, Johansen HT, Pettersen SJ, Solberg R, Brix K, et al. (2013) Nuclear legumain activity in colorectal cancer. PLoS One 8: e52980.
16. Wu B, Yin J, Texier C, Roussel M, Tan KS (2010) Blastocystis legumain is localized on the cell surface, and specific inhibition of its activity implicates a pro-survival role for the enzyme. J Biol Chem 285: 1790–1798.
17. Liu Z, Jang SW, Liu X, Cheng D, Peng J, et al. (2008) Neuroprotective actions of PIKE-L by inhibition of SET proteolytic degradation by asparagine endopeptidase. Mol Cell 29: 665–678.
18. Shirahama-Noda K, Yamamoto A, Sugihara K, Hashimoto N, Asano M, et al. (2003) Biosynthetic processing of cathepsins and lysosomal degradation are abolished in asparaginyl endopeptidase-deficient mice. J Biol Chem 278: 33194–33199.
19. Abisi S, Burnand KG, Humphries J, Waltham M, Taylor P, et al. (2008) Effect of statins on proteolytic activity in the wall of abdominal aortic aneurysms. Br J Surg 95: 333–337.
20. Wang ZH, Liu XL, Zhong M, Zhang LP, Shang YY, et al. (2010) Pleiotropic effects of atorvastatin on monocytes in atherosclerotic patients. J Clin Pharmacol 50: 311–319.
21. Halfon S, Patel S, Vega F, Zurawski S, Zurawski G (1998) Autocatalytic activation of human legumain at aspartic acid residues. FEBS Lett 438: 114–118.
22. Li DN, Matthews SP, Antoniou AN, Mazzeo D, Watts C (2003) Multistep autoactivation of asparaginyl endopeptidase in vitro and in vivo. J Biol Chem 278: 38980–38990.
23. Smith R, Johansen HT, Nilsen H, Haugen MH, Pettersen SJ, et al. (2012) Intra- and extracellular regulation of activity and processing of legumain by cystatin E/M. Biochimie 94: 2590–2599.
24. Moheimani F, Kim CH, Rahmanto AS, van Reyk DM, Davies MJ (2012) Inhibition of lysosomal function in macrophages incubated with elevated glucose concentrations: a potential contributory factor in diabetes-associated atherosclerosis. Atherosclerosis 223: 144–151.
25. Gaster M, Kristensen SR, Beck-Nielsen H, Schroder HD (2001) A cellular model system of differentiated human myotubes. APMIS 109: 735–744.
26. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.
27. Johansen HT, Knight CG, Barrett AJ (1999) Colorimetric and fluorimetric microplate assays for legumain and a staining reaction for detection of the enzyme after electrophoresis. Anal Biochem 273: 278–283.
28. Barrett AJ, Kirschke H (1981) Cathepsin B, Cathepsin H, and cathepsin L. Methods Enzymol 80: 535–561.
29. Werle B, Ebert W, Klein W, Spiess E (1995) Assessment of cathepsin L activity by use of the inhibitor CA-074 compared to cathepsin B activity in human lung tumor tissue. Biol Chem Hoppe Seyler 376: 157–164.
30. Wensaas AJ, Rustan AC, Lovstedt K, Kull B, Wikstrom S, et al. (2007) Cell-based multiwell assays for the detection of substrate accumulation and oxidation. J Lipid Res 48: 961–967.
31. Takaguri A, Satoh K, Itagaki M, Tokumitsu Y, Ichihara K (2008) Effects of atorvastatin and pravastatin on signal transduction related to glucose uptake in 3T3L1 adipocytes. J Pharmacol Sci 107: 80–89.
32. Aas V, Bakke SS, Feng YZ, Kase ET, Jensen J, et al. (2013) Are cultured human myotubes far from home? Cell Tissue Res.
33. Colella R, Roisen FJ, Bird JW (1986) mRNA levels of cathepsins B and D during myogenesis. Biomed Biochim Acta 45: 1413–1419.
34. Qian F, Chan SJ, Gong QM, Bajkowski AS, Steiner DF, et al. (1991) The expression of cathepsin B and other lysosomal proteinases in normal tissues and in tumors. Biomed Biochim Acta 50: 531–540.
35. Koh KK, Quon MJ, Han SH, Lee Y, Ahn JY, et al. (2008) Simvastatin improves flow-mediated dilation but reduces adiponectin levels and insulin sensitivity in hypercholesterolemic patients. Diabetes Care 31: 776–782.
36. Koh KK, Quon MJ, Han SH, Lee Y, Kim SJ, et al. (2010) Atorvastatin causes insulin resistance and increases ambient glycemia in hypercholesterolemic patients. J Am Coll Cardiol 55: 1209–1216.
37. Park SY, Choi GH, Choi HI, Ryu J, Jung CY, et al. (2005) Depletion of mitochondrial DNA causes impaired glucose utilization and insulin resistance in L6 GLUT4myc myocytes. J Biol Chem 280: 9855–9864.
38. Minet AD, Gaster M (2010) ATP synthesis is impaired in isolated mitochondria from myotubes established from type 2 diabetic subjects. Biochem Biophys Res Commun 402: 70–74.
39. Stevens TH, Forgac M (1997) Structure, function and regulation of the vacuolar (H+)-ATPase. Annu Rev Cell Dev Biol 13: 779–808.
40. Deng D, Jiang N, Hao SJ, Sun H, Zhang GJ (2009) Loss of membrane cholesterol influences lysosomal permeability to potassium ions and protons. Biochim Biophys Acta 1788: 470–476.
41. Jadot M, Andrianaivo F, Dubois F, Wattiaux R (2001) Effects of methylcyclodextrin on lysosomes. Eur J Biochem 268: 1392–1399.
42. Subtil A, Gaidarov I, Kobylarz K, Lampson MA, Keen JH, et al. (1999) Acute cholesterol depletion inhibits clathrin-coated pit budding. Proc Natl Acad Sci U S A 96: 6775–6780.
43. Pike LJ, Miller JM (1998) Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. J Biol Chem 273: 22298–22304.
44. Peres C, Yart A, Perret B, Salles JP, Raynal P (2003) Modulation of phosphoinositide 3-kinase activation by cholesterol level suggests a novel positive role for lipid rafts in lysophosphatidic acid signalling. FEBS Lett 534: 164–168.
45. Minogue S, Chu KM, Westover EJ, Covey DF, Hsuan JJ, et al. (2010) Relationship between phosphatidylinositol 4-phosphate synthesis, membrane organization, and lateral diffusion of PI4KIIalpha at the trans-Golgi network. J Lipid Res 51: 2314–2324.
46. Waugh MG, Minogue S, Chotai D, Berditchevski F, Hsuan JJ (2006) Lipid and peptide control of phosphatidylinositol 4-kinase IIalpha activity on Golgi-endosomal Rafts. J Biol Chem 281: 3757–3763.
47. Flint OP, Masters BA, Gregg RE, Durham SK (1997) HMG CoA reductase inhibitor-induced myotoxicity: pravastatin and lovastatin inhibit the geranylgeranylation of low-molecular-weight proteins in neonatal rat muscle cell culture. Toxicol Appl Pharmacol 145: 99–110.
48. Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10: 513–525.

49. Thompson PD, Clarkson P, Karas RH (2003) Statin-associated myopathy. JAMA 289: 1681–1690.
50. Skottheim IB, Gedde-Dahl A, Hejazifar S, Hoel K, Asberg A (2008) Statin induced myotoxicity: the lactone forms are more potent than the acid forms in human skeletal muscle cells in vitro. Eur J Pharm Sci 33: 317–325.
51. Itagaki M, Takaguri A, Kano S, Kaneta S, Ichihara K, et al. (2009) Possible mechanisms underlying statin-induced skeletal muscle toxicity in L6 fibroblasts and in rats. J Pharmacol Sci 109: 94–101.

Simvastatin inhibits glucose metabolism and legumain activity in human myotubes

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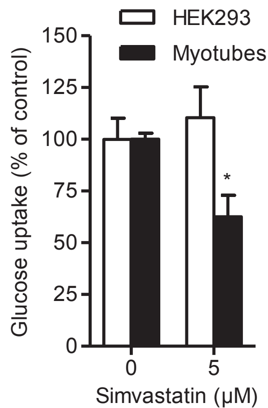
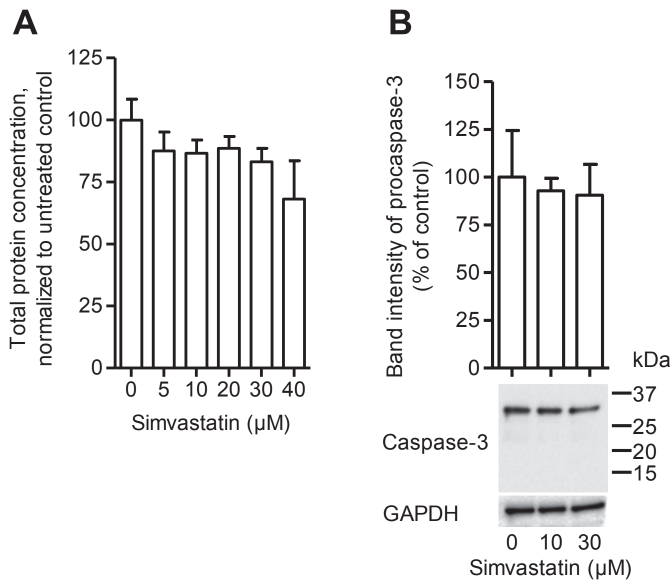
Supplementary data

Suppl. fig. 1



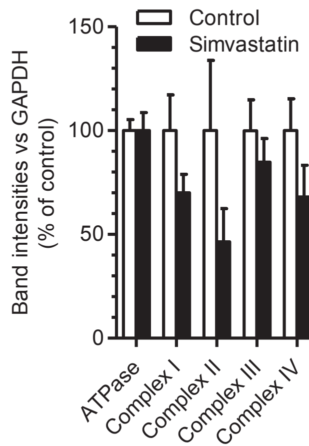
Supplementary figure 1. Simvastatin reduced glucose uptake in myotubes but not in HEK293 cells. Differentiated myotubes or HEK293 cells were incubated for 48 h with or without (C) 5 µM simvastatin prior to incubation for 4 h with [^{14}C(U)]glucose (0.2 mM, 21.5 kBq/ml) using a multiwell trapping device. Radioactivity was measured in cell lysates and corrected for total protein within each treatment (n=4-8, student t-test, *p<0.05 vs. untreated).

Suppl. fig. 2



Supplementary figure 2. Effects of simvastatin on total protein content (A) and caspase-3 expression (B) in differentiated myotubes. Differentiated myotubes were incubated for 48 h with or without simvastatin (5-40 µM). **A.** Total protein concentrations in cell lysates were measured and normalized to untreated control (n=17). **B.** Equal amounts of total proteins (10 µg) of cell lysates were analyzed for caspase-3 by immunoblotting. One representative immunoblot is shown and band intensity analysis of procaspase-3 are normalised to untreated control (n=3).

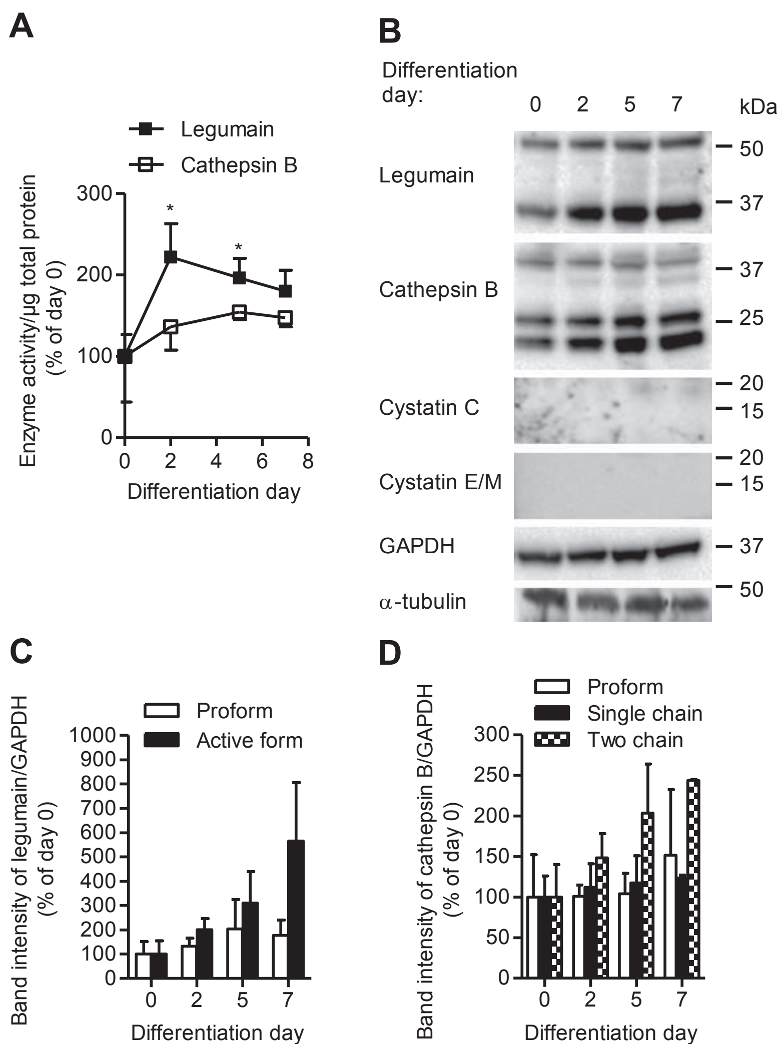
Suppl. fig. 3



Supplementary figure 3: Expression of ATPase, complex I, II, III and IV after simvastatin treatment.

Differentiated myotubes were pre-incubated for 48 h with simvastatin (10 or 30 µM; pooled results) prior to harvesting at day 7. Ten-twenty µg total protein were loaded per well and immunoblotting was perfomed. Antibodies against ATPase, complex I, II, III, IV and GAPDH were used. GAPDH was used as loading control. Quantification of immunoband intensities are shown, corrected for GAPDH and normalized to control (n=7).

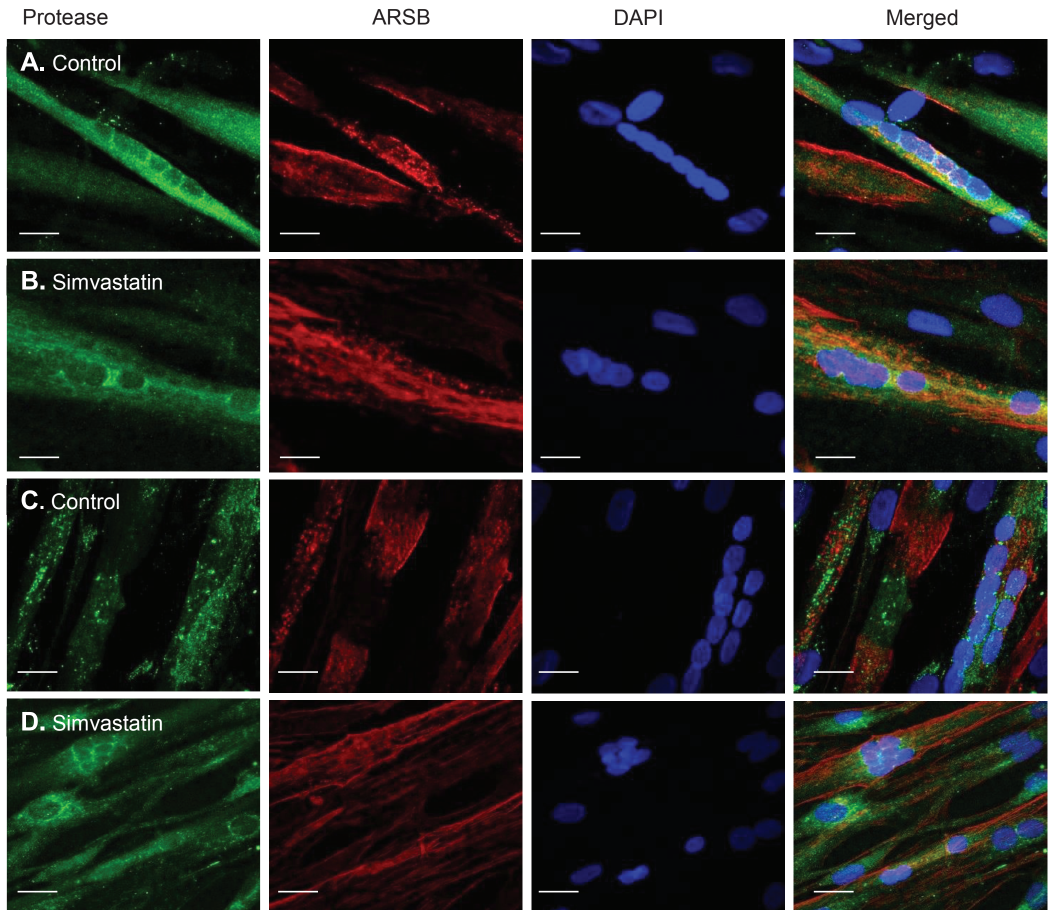
Suppl. fig. 4



Supplementary figure 4. Characterization of legumain and cathepsin B during differentiation of human myotubes.

Myoblasts (50,000 cells/well) were cultured and proliferated to 80-90 % confluence before start of differentiation (day 0), and cells were harvested as indicated at day 0, 2, 5 and 7. **A**. Legumain and cathepsin B activities in cell lysates were measured by cleavage of fluorogenic peptide substrates (n=3-4, student t-test, *p<0.05 vs. differentiation day 0). **B**. Representative immunoblots of cell lysates are shown. Equal amounts of total proteins (10 µg/well) were applied to the gel and immunoblot analysis were performed (Rh.leg., recombinant human legumain (5 ng)). Band intensity of legumain (C) and cathepsin B (D) was measured and normalized to differentiation day 0 (n=3).

Suppl. fig 5



Supplementary figure 5. Localisation of legumain and cathepsin B in myotubes treated with or without simvastatin.
Myotubes were cultured, differentiated, and incubated for 48 h without (control) or with 30 μM simvastatin prior to fixation at day 7. After fixation, the cells were permeabilized, blocked and further incubated with primary antibodies against legumain (green; A and B) or cathepsin B (green; C and D) and arylsulfatase B (ARSB; red). Secondary antibodies against the species of the primary antibodies and DAPI (blue) were used. The cells were photographed with identical camera and laser settings by LSM710 confocal microscopy (scale bars, 20 μm).

IV