Proteolytic Enzymes as Therapeutic Targets

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Dr Urs Lüthi carried out his PhD studies at ESBATech under the supervision of Dr Alcide Barberis and has recently completed his dissertation on the subject of functional screening systems in yeast to identify secretases, targets of secretases and modulators of secretase activity. An essential part of his work focused on the development of the cellular assay for BACE activity that is further developed for small compound inhibitors screens. He will continue to develop his expertise as a Senior Scientist with ESBATech and will be responsible for the development of similar cell-based assay systems. Urs' academic work was presented at several international conferences and published in peer-reviewed journals.

The regulation of protein secretion is central to the proper functioning of eukaryotic organisms. One mode of protein secretion is the generation of soluble fragments of membrane-bound proteins by limited proteolysis through the action of proteases termed secretases, or sheddases. In general, cleavage occurs close to the extracellular face of the membrane, releasing active protein. Proteins secreted in this fashion include some membrane receptors and receptor ligands, ectoenzymes and cell adhesion molecules. Since the proteins concerned are involved in pathophysiological processes, such as neurodegeneration, inflammation and oncogenesis, inhibitors of respective secretases are emerging as promising therapeutic drugs. This article will review a number of methods that have allowed molecular cloning of secretases and discuss different approaches to discovering specific secretase inhibitors.

Today, inhibitors against HIV-1 protease (to treat acquired immunodeficiency syndrome [AIDS]) and against the angiotensin-converting enzyme (ACE) (to treat hypertension) are the only protease inhibitors approved by the FDA for therapeutic use (see Table 1). HIV-1 protease is an aspartic protease that is necessary for the processing of viral polypeptides late in the viral replicative cycle. Inhibition of this activity prevents formation of functional viral peptides and, thus, formation of infectious virions (1). The ACE, a zinc metalloprotease, controls formation of angiotensin II by proteolytic processing of

Table 1: Examples of Proteases, Inhibitors and their Clinical Status

Protease	Function	Disease	Inhibitor and Status
HIV-1 protease	Viral replication	AIDS	Saquinavir (Roche), ritonavir (Abbott), indinavir (Merck), nelfinavir (Pfizer/Agouron), amprenavir (Vertex/Glaxo Wellcome) FDA approved for therapeutic use
Angiotensin-converting enzyme (ACE)	Generation of angiotensin II	Hypertension, congestive heart failure	Captopril, enalapril and enalaprilat, lisinopril, benazepril, moexipril, trandolapril, fosinopril, ramipril, quinapril FDA approved for therapeutic use
Rhinovirus 3C protease	Viral replication	Common cold	Ag7088; Phase II
Proteasome	Protein degradation	Colon, breast, lung cancer	PS-341; Phase II
Tryptase inhibitor	Inflammation	Psoriasis, acute inflammatory bowel disease	APC-2059; Phase III
Hepatitis C virus NS3 protease	Viral replication	Hepatitis	VX-950; preclinical
Thrombin	Blood coagulation	Stroke	Ximelagatran; Phase III
ACE + neutral endopeptidase	Generation of angiotensin II	High blood pressure, congestive heart failure	Omapatrilat ACE/NEP inhibitor 100/240; Phase II
ICE (caspase-1)	Conversion of interleukin-1_	Rheumatoid arthritis, osteoarthritis	Pralnacasan 3840/VX-740; phase II VX-765; preclinical
Caspases	Apoptosis and inflammation	Sepsis	VX-799; preclinical

its precursor decapeptide angiotensin I. Angiotensin II causes potent vasoconstriction via binding to G-protein coupled receptors. Inhibition of ACE activity decreases angiotensin II levels, thereby leading to a decrease in blood pressure. More than 10 ACE inhibitors have been approved by the FDA for therapeutic use (2). Inhibitors against these two proteases represent the first successful examples of rationally designed drugs. Their design and launch in the 1990s provoked a boost in research in the protease field. Molecular cloning of novel proteases playing important roles in cellular processes shifted the perception of their function. Today, proteases are no longer thought of as simply destructive enzymes, but are being recognised as key players in the regulation of many physiological processes.

Proteases are classified according to their catalytic mechanism into aspartic, cysteine (thiol), serine and metalloproteases (3). The database MEROPS (www.merops.co.uk) currently lists over 400 human sequences, which stand at 3 per cent aspartic, 23 per cent cysteine, 32 per cent serine and 36 per cent metalloproteases. Cysteine and serine proteases proceed catalysis in much the same manner through the formation of a covalent intermediate involving a catalytic triad (his, asp, ser residues) for serine and a cysteine residue for thiol proteases. The majority of metalloproteases contain the sequence HEXXH, which provides two histidine ligands for the binding of a zinc atom in the active site. Aspartic proteases, such as members of the pepsin family, are structurally bi-lobed molecules with the active site located between two lobes. Each lobe contributes one aspartate residue to the catalytic dyad.

Proteases that cleave membrane proteins of type-I or type-II topology to generate circulating, soluble forms are referred to as 'secretases' or 'sheddases'. In general, secretases themselves are also bound to membranes either through a transmembrane domain or by a glycosyl-phosphatidylinositol (GPI) anchor. Cleavage by secretases occurs close to the extracellular face of the membrane or within the transmembrane domain, as has been demonstrated for the cleavage of the amyloid precursor protein (APP) by the so-called γ-secretase (4). Further examples of secretase substrates are tumour-necrosis factor (TNF)-α, fas ligand, transforming growth factor (TGF)-α, TNF receptor-1 and -2, the interleukin (IL) receptor-1, nerve growth factor (NGF) receptor, platelet-derived growth factor (PDGF) receptor and others.

IDENTIFICATION AND CHARACTERISATION OF SECRETASES

Traditional Approaches

Most soluble proteases have been cloned by exploiting the sequence-specificity of their proteolytic activity for biochemical purification. The purified fraction was then partially sequenced by Edman degradation. The derived sequences were used to design oligonucleotide probes for the identification and isolation of the respective cDNA clone by convential library screening. This traditional approach was also successfully applied for the cloning of some membrane-bound secretases, such as the TNF-α converting enzyme TACE (5,6) and the disintegrin-metalloprotease MADM (ADAM10) (7).

Nevertheless, different approaches had to be taken to clone other secretases, which could not be readily identified by the traditional approach.

- Presenilin-1, an eight-pass transmembrane protein that is responsible for a very aggressive form of Alzheimer's disease when mutated, was cloned by genetic linkage analysis (8). Such a genetic approach is very laborious, since it requires detailed tracing of co-segregation of the disease phenotype with genetic markers.

- The β-secretase BACE, also causally involved in the development of Alzheimer's disease, was identified by an expression cloning strategy (9). BACE encodes a type-I aspartic secretase, which processes the amyloid precursor protein, releasing an ectodomain of unknown function. Several thousand pools, each containing a limited number of cDNAs, were transfected into a cell line that overexpressed APP. Processing of APP was measured for each transfection, and positive pools were subsequently subdivided into smaller pools and re-tested for APP cleavage upon transfection. This stepwise approach resulted in the isolation of a single cDNA clone (9).

- Most matrix metalloproteases (MMPs) were cloned by degenerate oligonucleotide primers that were

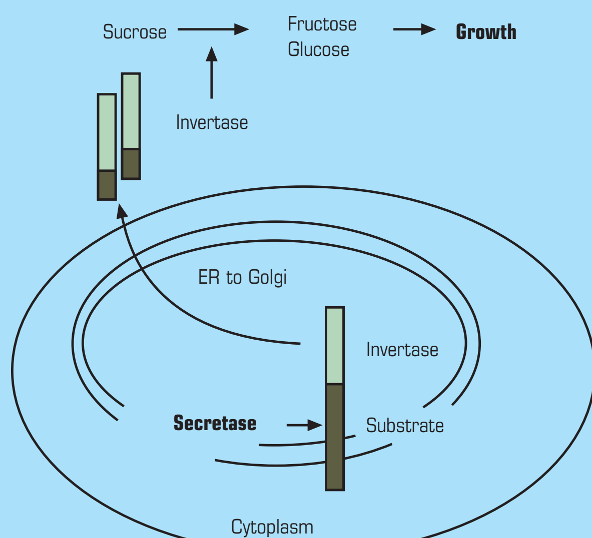
specific for highly conserved sequences found in all MMPs. Matrix metalloproteases comprise a family of over 20 enzymes that are not only involved in the degradation of the extracellular matrix, but can also function as secretases (10). For example, MMP-2 has been shown to cleave fibroblast growth factor (FGF) receptor-1 at the cell surface, thereby releasing an active soluble ectodomain (11).

The involvement of secretases in many pathophysiological processes makes this class of enzymes an attractive target for the development of therapeutic drugs. However, to date, only a percentage of secretases has been identified. Efficient cleavage of secretase substrates requires, at least in some cases, membrane insertion and the use of the whole protein, as opposed to short peptides. Therefore, traditional approaches comprising purification and assaying for secretase activity may be inappropriate for certain secretases. This raises the need for reliable screening systems, which are more efficient than known traditional methods, for the cloning of new members of the secretase family. The exploitation of yeast versatility for developing expression cloning systems provides an alternative.

Figure 1: Cell Growth Selection System in Yeast to Identify Secretases and Cytosolic Proteases

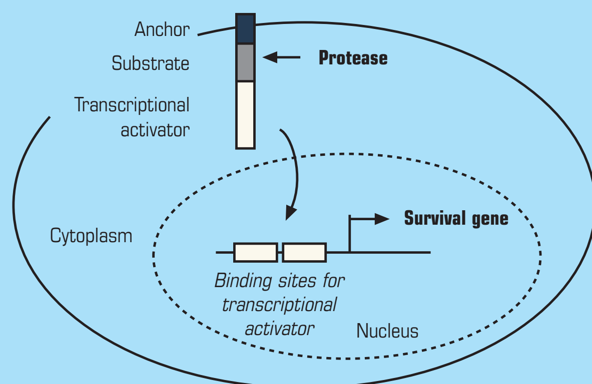
a) The secretase substrate is expressed as a fusion protein with the yeast enzyme invertase and localised as membrane protein to ER and Golgi compartments. Cleavage by a specific secretase derived from a co-expressed cDNA library releases the invertase moiety, which, upon secretion to the periplasmic space, hydrolyses sucrose to yield fructose and glucose. Therefore, only secreted invertase allows colony formation on plates containing sucrose as the sole carbon source.

Figure 1a



b) A cytoplasmatically located transcriptional activator is targeted to intracellular membranes via a membrane anchor. The protease substrate including the cleavage site is inserted between the two domains. Sequence-specific protease activity derived from a co-expressed cDNA library releases the activator, which then migrates to the nucleus and activates transcription of a survival gene, thereby allowing cell growth.

Figure 1b

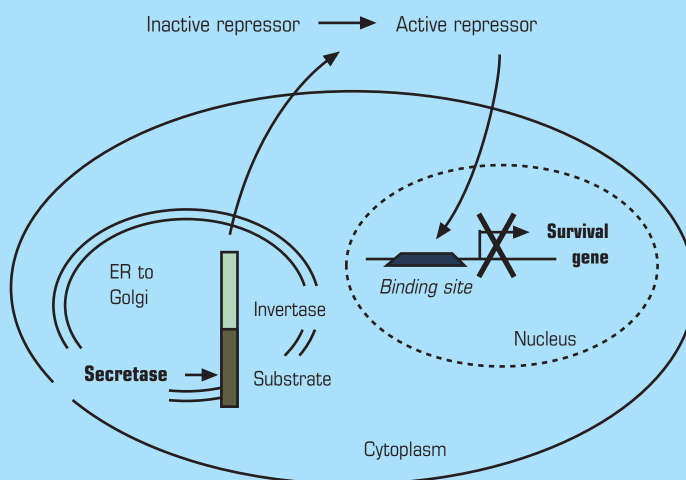


A Cellular System to Identify Secretases

A yeast cell represents the prototype of a eukaryotic cell. It displays a similar cellular architecture as a human cell, but cultivation and genetic manipulations are simpler and faster. This makes yeast a valuable tool for *in vivo* screening systems. We have established a cell growth selection system in the yeast *S. cerevisiae* that allows screening for secretase activity. This system is based on the ability of yeast to utilise sucrose as a carbon source and its dependence on the secretion of the enzyme invertase, which cleaves sucrose to yield glucose and fructose (12). Disruption of the *SUC2* gene, which encodes invertase, cripples the ability of yeast cells to grow on sucrose medium. To achieve cell growth in the presence of an active secretase, the invertase is fused to a secretase substrate, which is anchored to the membrane via its transmembrane domain (see Figure 1a). The invertase moiety is localised in the secretory pathway, but significant amounts of invertase activity at the cell surface are prevented by retention of the fusion protein in the endoplasmatic reticulum (ER) and Golgi. Human secretases that are expressed from a cDNA library and that can cleave the invertase fusion protein within the luminal domain of the substrate, cause liberation and subsequent secretion of the invertase moiety. Invertase activity at the cell surface allows cell growth on sucrose plates. Thus, co-expression of substrate-specific human secretases leads to the formation of yeast colonies.

Yeast genetics can also be exploited to screen for secretases that cleave their targets within the transmembrane domain or for cytosolic proteases. In this case, the substrate is expressed as a fusion protein with a cytoplasmatically located transcriptional activator (see Figure 1b). Substrate-specific proteolytic activity releases

Figure 2: Yeast Selection System for Identifying Secretase Inhibitors



This system is a further developed version of the invertase-based selection system described in Figure 1a. Cleavage of the substrate by a functional secretase causes release and subsequent secretion of the invertase moiety. Secreted invertase activity produces an active repressor, which is internalised and represses transcription of a survival gene resulting in growth inhibition. However, a compound that inhibits secretase activity prevents invertase secretion and therefore formation of the active repressor. In this case, the survival gene is constitutively transcribed allowing cells to grow.

the transcription factor, which translocates to the nucleus and activates expression of a survival gene, thereby allowing cell growth. Successful application of this system has been shown by Steiner *et al* (13).

IDENTIFICATION OF PROTEASE INHIBITORS

Proteolytic activity is not only required for maintenance of normal cellular functions but is also central to the pathogenesis of a variety of human diseases. Parasitic (for example schistosomiasis and malaria), fungal (such as *C. albicans*) and viral infections (for example HIV, herpes and hepatitis) but also cancer, inflammatory, respiratory, cardiovascular and neurodegenerative diseases, including Alzheimer's, require proteolytic activity for progression. Therefore, proteases and secretases involved in these diseases are important therapeutic targets (14).

Over the last 30 years, methods for the isolation of protease inhibitors have evolved from mass screening of natural and synthetic product libraries to analogue-based drug design, and more recently has advanced further to computer-assisted rational drug design. The latter approach, which requires knowledge of the three-dimensional structure of the protease, allows prediction of an interaction between the protease and a compound at the atomic level. This permits rational development of structurally distinct compounds with differing pharmacological profiles. For the clinical status of selected inhibitors see Table 1.

Successful application of protease inhibitors in human therapy requires defined properties of the drug, such as membrane permeability, stability and the lack of toxicity. Cell-free screening systems consist of purified, recombinant protease – in case of secretases truncated versions lacking the transmembrane domain are used – and a synthetic substrate, the cleavage of which is detected by biochemical methods. Such screening systems have major limitations, since compounds are selected only for inhibition of *in vitro* proteolytic activity, but not for membrane permeability, stability and cell toxicity. An alternative to such cell-free systems is provided by cellular screening systems, which positively select compounds for inhibition of protease activity and negatively select compounds that are toxic in a cellular environment, or unstable, or that show low membrane permeability. Furthermore, expression of membrane-anchored target proteins allows screening for inhibitors of secretase activity directed to its substrate in a natural conformation.

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Yeast can be exploited to perform functional screenings for secretase inhibitors in a eukaryotic environment. The invertase-based selection system, as outlined in Figure 1a (page 90), would be inappropriate because inhibited secretase activity results in growth inhibition. To achieve cell growth in the presence of a specific inhibitor, this selection system was modified as follows (see Figure 2): a secretase that cleaves the substrate causes release and subsequent secretion of the invertase moiety. In the medium, an inactive repressor molecule is converted to an active repressor upon processing by secreted invertase activity. The active repressor is internalised and represses transcription of a survival gene, resulting in growth inhibition. However, the presence of a secretase inhibitor prevents display of invertase activity at the cell surface and, thus, formation of the active repressor. In this case, the survival gene is transcribed in a constitutive manner, thereby allowing colony formation on plates or, alternatively, cell growth in liquid medium.

CONCLUSION

The inhibition of HIV-1 protease and ACE demonstrated the potential of protease inhibitors in the treatment of viral infections and cardiovascular diseases. The involvement of defined proteolytic activities in the progression of many diseases turns proteases into attractive therapeutic targets. The challenge for the pharmaceutical industry is to design specific inhibitors that target discrete proteases. Potent, selective inhibitors will only be developed by a combination of different methods, including mass screening of libraries, rational drug design and computing technology. Cellular systems provide promising tools to screen for inhibitors in *de novo* drug design, particularly if the three-dimensional structure of the target protein is not yet determined at the atomic level. ◆

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References

(1) Tomasselli AG and Heinrikson RL, Targeting the HIV-protease in AIDS therapy: a current clinical perspective, *Biochim Biophys Acta*, 1477(1-2): pp189-214, 2000

(2) Hilleman DE, Role of angiotensin-converting-enzyme inhibitors in the treatment of hypertension, *Am J Health Syst Pharm*, 57 Suppl 1: pp8-11, 2000

(3) Barrett AJ, Rawlings ND and Woessner JF, *Handbook of Proteolytic Enzymes*, 1998

(4) Selkoe DJ, Alzheimer's disease: genes, proteins, and therapy, *Physiol Rev*, 81(2): pp741-66, 2001

(5) Black RA *et al*, A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells, *Nature*, 385(6618): pp729-33, 1997

(6) Moss ML *et al*, Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha [published erratum appears in *Nature*, 386(6626):738], *Nature*, 85(6618): pp733-6, 1997

(7) Howard L *et al*, Molecular cloning of MADM: a catalytically active mammalian disintegrin-metalloprotease expressed in various cell types, *Biochem J*, 317(Pt 1): pp45-50, 1996

(8) Sherrington R *et al*, Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease (see comments), *Nature*, 375(6534): pp754-60, 1995

(9) Vassar R *et al*, Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE (see comments), *Science*, 286(5440): pp735-41, 1999

(10) McCawley LJ and Matrisian LM, Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol*, 13(5): pp534-40, 2001

(11) Levi E *et al*, Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1, *Proc Natl Acad Sci U S A*, 93(14): pp7,069-74, 1996

(12) Carlson M *et al*, The secreted form of invertase in saccharomyces cerevisiae is synthesized from mRNA encoding a signal sequence, *Mol Cell Biol*, 3(3): pp439-47, 1983

(13) Steiner H, Pesold B and Haass C, An *in vivo* assay for the identification of target proteases which cleave membrane-associated substrates, *FEBS Lett*, 463(3): pp245-9, 1999

(14) Hooper NM, Karran EH and Turner AJ, Membrane protein secretases, *Biochemical Journal*, 321(Pt 2): pp265-79, 1997

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