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Interaction Studies of Secreted Aspartic Proteases (Saps) from *Candida albicans*

Application for Drug Discovery

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Abstract
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This thesis is focused on enzymatic studies of the secreted aspartic proteases (Saps) from *Candida albicans* as a tool for discovery of anti-*candida* drugs. *C. albicans* causes infections in a number of different locations, which differ widely in the protein substrates available and pH. Since *C. albicans* needs Saps during virulent growth, these enzymes are good targets for drug development.

In order to investigate the catalytic characteristics of Saps and their inhibitor affinities, substrate-based kinetic assays were developed. Due to the low sensitivity of these assays, especially at the sub-optimal pH required to mimic the different locations of infections, these assays were not satisfactory. Therefore, a biosensor assay was developed whereby, it was possible to study interaction between Saps and inhibitors without the need to optimise catalytic efficacy. Furthermore, the biosensor assay allowed determination of affinity, as well as the individual association and dissociation rates for inhibitor interactions.

Knowledge about substrate specificity, Sap subsite adaptivity, and the pH dependencies of catalytic efficacy has been accumulated. Also, screening of transition-state analogue inhibitors designed for HIV-1 protease has revealed inhibitors with affinity for Saps. Furthermore, the kinetics and pH dependencies of their interaction with Saps have been investigated. One of these inhibitors, BEA-440, displayed a complex interaction with Saps, indicating a conformational change upon binding and a very slow dissociation rate. A time dependent interaction was further supported by inhibition measurements. The structural information obtained affords possibilities for design of new more potent inhibitors that might ultimately become drugs against candidiasis. The strategy to combine substrate specificity studies with inhibitor screening has led to complementary results that generate a framework for further development of potent inhibitors.

Keywords: SPR Biosensor, Secreted aspartic proteases, Candida albicans, interaction kinetics, drug discovery, protease inhibitor

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I dedicate this thesis to all the people
that fight every day to survive AIDS,
only to acquire recurring candidiasis.

List of papers

I Rubin, R., Beyer, B.B., Chung A.Y., Johnson, J.V., Backman, D., Danielson, U.H., and Dunn, B.M. Discovery of the subsite specificity of *Candida albicans* Sap1 and Sap2 and the design of potent peptidomimetic inhibitors (Manuscript)

II Backman, D. and Danielson, U.H. Substrate and pH dependence of secreted aspartic proteases Sap1, Sap2 and Sap3 from *Candida albicans* (Manuscript)

III Backman, D. and Danielson, U.H. (2003) Kinetic and mechanistic analysis of the association and dissociation of inhibitors interacting with secreted aspartic proteases 1 and 2 from *Candida albicans*. *Biochimica et Biophysica Acta* **1646** (1-2), 184-195.

IV Backman, D., Monod, M. and Danielson, U.H. Biosensor-based screening and characterization of HIV-1 inhibitor interactions with Sap1, Sap2 and Sap3 from *Candida albicans* (Submitted)

Contents

Abbreviations

ADME	Adsorption, Distribution, Metabolism and Excretion
AIDS	Acquired immunodeficiency syndrome
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
$\Delta G°$	Gibbs free energy
HAART-PI	Highly active anti retroviral therapy-protease inhibitors
K_D	Equilibrium dissociation constant
K_i	Equilibrium inhibition constant
K_M	Michaelis constant
k_{cat}	Turnover number
k_{on}	Association rate constant
k_{off}	Dissociation rate constant
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
RHE	Reconstructed human epitelium
RNA	Ribonucleic acid
SAP	Secreted aspartic proteases
SPR	Surface plasmon resonance

Introduction

Candida albicans

Candida albicans is a polymorphic opportunistic fungal pathogen and the major cause of candidiasis in humans. It can adopt three different morphological growth patterns, hyphal, pseudohyphal and yeast growth. Many people are carriers of this fungus but it only causes infections in relatively few individuals.

Often infections are localized to the skin, gastrointestinal tract or in the oral or vaginal cavity without causing severe infections. However, in people with reduced immune responses due to cancer or transplantation therapy or AIDS, it can cause a severe systemic candidiasis that is lethal. About 75% of all healthy women will experience at least one vaginal yeast infection and about 5% get recurrent infections (*Sobel et al.* 1988, *Sobel et al.* 1992). Some 80-95% of HIV-infected patients will have at least one episode of oropharyngeal candidiasis (*Borg-von Zepelin et al.* 1999). Other infectious *Candida species* include *C. tropicalis, C. parapsilosis and C. lusitaniae*.

Treatment of candidiasis

There is a limited number of drugs against candidiasis (*Rogers et al.* 2001). It is often treated with azole drugs such as fluconazole, itrakonazole and ketoconazole. Other anti-*Candida* drugs include flucytosine and amphotericin B. Since the eighties, fungal infections have become much more frequent (*Odds et al* 2003) and the use of these drugs has resulted in decreased susceptibilities and resistance, towards these drugs.

The target for azole-based drugs is a cytochrome P450 enzyme that catalyses the removal of the 14α-methyl group of lanosterol thereby interfering with the ergosterol biosynthetic pathway and thus interfering with normal synthesis of membrane structure. Resistance against azole drugs can occur by mutations in the target enzyme or by overexpression of efflux pumps that export antifungals from the cell. In addition, combinations of these two mechanisms have been reported (*Odds et al* 2003).

Flucytosine interferes with the RNA polymerase of the fungus and causes premature chain termination in *Candida*. However, it also causes several side-effects probably due to similarities between the *Candida* and human RNA polymerases. Amphotericin B binds to ergosterol, thereby preventing

normal membrane function and causing leakage of the cell contents. Unfortunately, this mechanism of action is also seen in humans, where this drug causes nephrotoxicity (*Odds et al* 2003). Resistance and decreased susceptibility development seems to be much slower and more rare for amphotericin B compared to azole-based drugs, possibly because there is no possibility of changing the target.

Treatment with a combination of an azole drug and amphotericin B has been successful both in clearance of *Candida* infections and as a way to minimize the spread of decreased susceptibility and resistance. Combination therapy in the treatment of HIV has been successful in combating the appearance of resistance. However, sooner or later resistance will cause decreased efficacy of the drugs.

For *Candida,* the development of resistance takes longer than for HIV. This extra time should be used to find new and effective treatments before multi-resistant fungal infections emerge. In general antifungal drugs should have a broad specificity against several different fungal pathogens.

Enzymes as drug targets

Historically, drugs have been designed on a trial-and-error basis, without the actual knowledge of how they work. Today, strategies based on targeting a receptor or an enzyme has made possible a more focused approach to drug discovery. HIV therapy is a success story for target based drug discovery. Both the protease and the reverse transcriptase have been used as drug targets, prolonging the lives of millions of HIV-infected patients.

The most important thing to consider when trying to target an enzyme is whether blocking the activity of the enzyme really results in the desired effect. This is called target validation. So in the case of infectious diseases, the goal must be that the enzyme used as a target is required by the infectious organism, either for survival or infection. Otherwise, the drug will only show effect *in vitro* and give no clearance of infection, and will therefore be useless.

This might seem like a trivial thing, but the hunt for plasmepsin I and II (aspartic proteases from *Plasmodium falciparum*) inhibitors as a method to develop drugs against malaria is an example of how things can go wrong. Originally, scientists working in this area thought that plasmepsins were responsible for the initial degradation of haemoglobin. However, it was later shown (*Shenai et al.* 2000*)* that also falcipains (cysteine proteases from *Plasmodium falciparum*) could degrade haemoglobin in a reducing environment. So the high efficacy of plasmepsin I and plasmepsin II inhibitors *in vitro*, did not show the desired correlation with the effects in cell culture. Whether this was due to residual proteolytic activity of plasmepsins not affected by these inhibitors, or whether falcipains have an overlapping activity, or if the transport of inhibitor into the food vacuole is inefficient is not

known. The fact that blocking this target did not correlate with cell assay data caused some drug companies to stop their malarial projects. Much work and a lot of money could have been saved if these targets had been better validated.

Proteases

Proteases or peptidases are enzymes that hydrolyse peptide bonds (of proteins or peptides) (Figure1). Generally one distinguishes among different types of proteases on the basis of their catalytic mechanism. There are four major groups of proteases; serine proteases, cysteine (thiol) proteases, metalloproteases and aspartic proteases.

The serine proteases have a catalytic triad of serine, aspartate and histidine. Serine proteases include well-known human proteases such as chymotrypsin and trypsin. The cysteine proteases are similar to the serine proteases but with a cysteine instead of a serine in the catalytic triad. Important cysteine proteases include human cathepsin S, postulated to be involved in a variety of diseases and falcipains from *Plasmodium falciparum*. The metalloproteases have a catalytically active metal ion bound to it as a cofactor. Metalloproteases include the large group of human matrix metalloproteases, involved in a variety of diseases.

Aspartic proteases

Aspartic proteases have two catalytic aspartate residues. To be catalytically active, the mechanism demands that one of the aspartates is protonated and the other is deprotonated, so the catalytically active enzyme has a negative charge in the active site. This is the reason for the fact that aspartic proteases have an acidic pH optimum (pH 2-6).

The mechanism of peptide hydrolysis (Figure1) is initiated by the abstraction of a proton from water by one of the aspartates, The produced hydroxide ion engages in a nucleophilic attack on the carbonyl carbon of the sicissle bond. This attack gives rise to the tetrahedral transition state, which is very unstable and decomposes to give the two resulting peptides and the regenerated protease (Figure1). Well-known aspartic proteases are the HIV-1 protease, human renin, pepsin and cathepsin D. The majority of the aspartic proteases are monomeric, but dimeric aspartic proteases exist, for example, the HIV-1 protease.

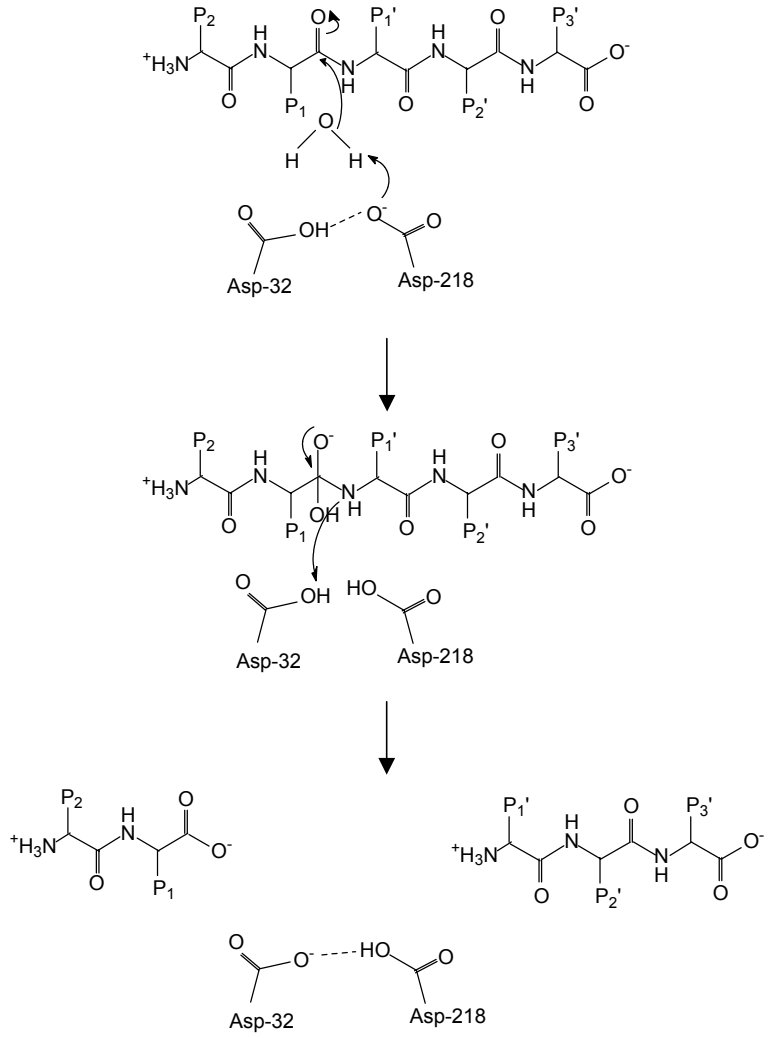

Figure 1. The catalytic mechanism for aspartic proteases (Sap2 numbering).

Enzyme kinetics and substrate specificity

Enzymes catalyze reactions and this is what one likes to study as an enzymologist. To do so one must have not only the enzymes, but also suitable substrates that enzymes can convert in to products, and it must be possible to measure the concentration of substrate or product. So when measuring rates of catalysis one either follows product formation or substrate disappearance.

A model peptide in the active site of a protease is shown in figure 2. The amino acid residues at the amino terminal part of the peptide are denoted P1,

P2, P3 etc from the cleavage site, according to the nomenclature for protease active sites and substrates by Schechter and Berger (1967). Towards the carboxyl terminal of the substrate amino acid residues are denoted P1', P2', P3' etc. The subsite in the protease that interacts with the different P positions are denoted in the same way, except with S instead of P; S5, S4, S3, S2, S1-S1', S2', S3'.

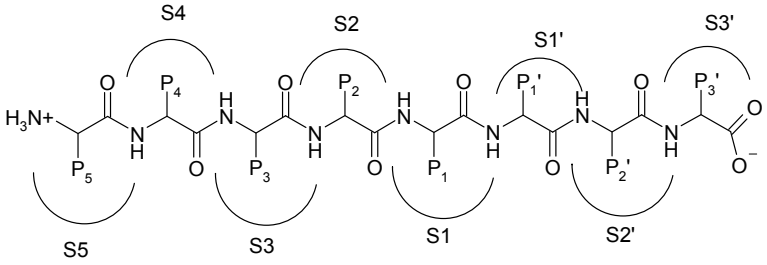

Figure 2. A model protease substrate S denotes the different subsites in the enzyme and P the different amino acid residues in the substrate (*Schechter et al.* 1967).

When determining catalytic constants for enzymes during the steady-state there is one important thing to consider. Does the reaction follow Henri-Michaelis-Menten kinetics (*Michaelis et al.* 1913)? If it does, one constant that is of interest is K_M, which in the simplest case is the dissociation constant (K_S) for the enzyme-substrate complex (Figure 3). If the product release is rate-limiting the K_M will be lower than the K_S. The turnover number (k_{cat}) gives a measure of how many substrate molecules that can be turned over to product by one enzyme molecule per unit time. The specificity constant (k_{cat}/K_M) is the constant to use when comparing substrate or enzyme specificity. In order to determine these constants, the Henri-Michaelis-Menten equation can be fitted to a set of data obtained by varying the substrate concentration and keeping the enzyme concentration constant (Figure 4).

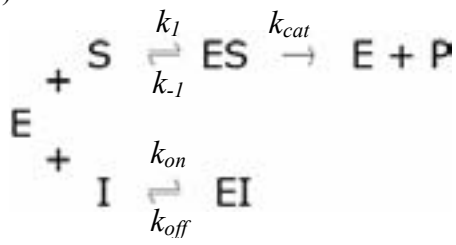

Figure 3. Reaction scheme with one substrate enzymatic reaction and a competivite inhibitor. In this simple case the K_M will be the dissociation constant for the enzyme-substrate complex. In a direct binding assay the enzyme-inhibitor equilibrium is studied, in contrast to inhibition studies were also the enzyme-substrate eqilibrium and catalytic step is studied. K_i is the dissociation constant for the enzyme-inhibitor complex.

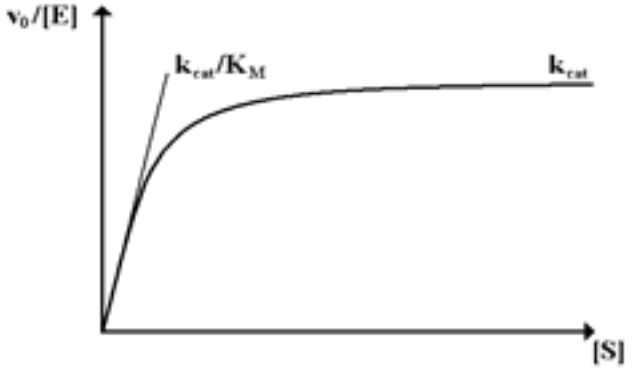

Figure 4. An ideal plot of initial reaction rate divided by enzyme concentration versus substrate concentration, representing the Michaelis-Menten equation $v_0/[E]=(k_{cat}*[S])/K_M+[S]$.

Transition-state analogue inhibitors of aspartic proteases

The inhibitors that have been studied in this project are transition-state analogues of aspartic proteases. According to transition-state theory the transition state is the state where bonds are broken and made, the highest energy state during the reaction. A transition-state analogue is a molecule that closely resembles the transition-state of the catalytic mechanism. For catalysis to be efficient the substrate has to bind with reasonable affinity and the transition state must bind with a much higher affinity.

So the transition state analogue must resemble the transition state, but should only bind to the active site and not turn over to product. Transition-state analogue inhibitors are typically more efficient than substrate analogue inhibitors since the transition state is bound with a much higher affinity. In the case of aspartic proteases, transition-state analogues should resemble the tetrahedral transition-state in the mechanism illustrated above (Figure 1). A number of chemical moieties have been used to mimic the transition state without becoming hydrolysed, in this study compounds with hydroxyethylene and dihydroxyethylene have been studied (Figure 5).

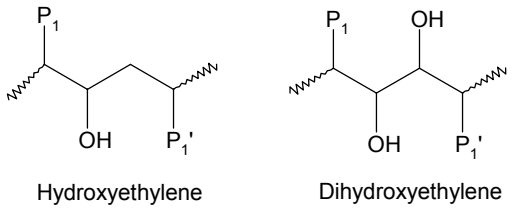

Figure 5. Transition-state isosteres used for protease inhibitor design.

Secreted aspartic proteases

C. albicans produces and secretes a family of aspartic proteases into their environment. The complete *C. albicans* genome is now sequenced (*Jones et al.* 2004) and ten different Saps have been found, they are denoted Sap1, Sap2 etc. SAP genes are translated as preproSaps into the ER (endoplasmatic reticulum). The signal peptide is processed by the signal peptidase in the ER and then transported to the golgi apparatus. In the golgi apparatus the Kex2 protease activates the Saps by proteolytic hydrolysis of the preSap which are subsequently secreted into the extra cellular space by the fungi (*Newport et al.* 1997). However, alternative processing pathways have also been suggested (*Togni et al.*1996)

The Saps are used by the fungus to degrade foreign tissue, for evading host defence and for acquiring amino acids that are needed for metabolism. The Saps biological substrates are numerous and include human serum albumin, keratin, collagen, salivary lactoferrin and immunoglobulins, including secretory IgA, which is normally resistant to most bacterial proteases. Like all aspartic proteases Saps are most active in acidic environments. The pH optimums for Sap1-3, the enzymes in focus in these studies, are between pH 3.3 and pH 4.5 (*Borg-von Zepelin et al.* 1998; *Koelsch et al.*2000).

The three-dimensional structures of Sap2 from *C. albicans* (Figure6) (*Cutfield et al.* 1995; *Abad-Zapatero et al.* 1996) and of Sapt from *C. tropicalis* (*Foundling et al.* 1997) have been determined by X-ray crystallography. The protein is a monomer with the typical aspartic acid protease structure.

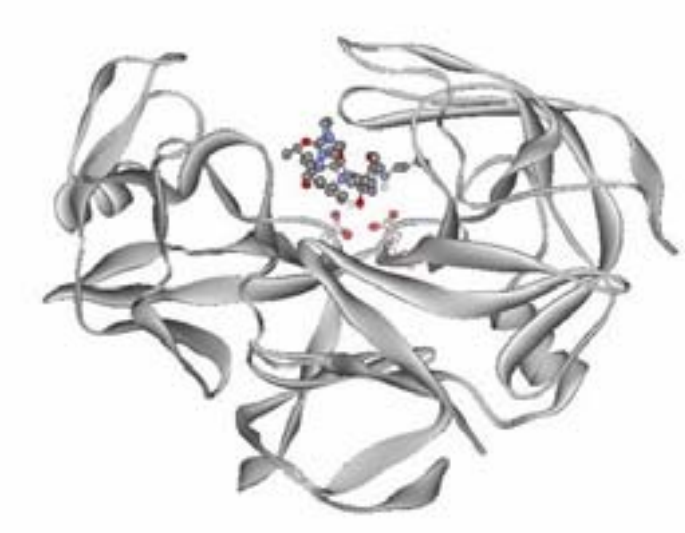

Figure 6. The structure of Sap2 (PDB 1ZAP), with an inhibitor (A-70450) bound to the active site. The inhibitor and the catalytic aspartates are in ball and stick representation.

Saps as virulence factors

Saps are one of the so-called virulence factors produced by the fungi. A virulence factor is a factor that is needed for infection. Other virulence factors that *C. albicans* have are hyphal formation, surface recognition molecules and phenotypic switching. A good review on Sap virulence is recommended for further reading (*Naglik et al.* 2003). The reasons for stating that Saps are virulence factors and that they are good targets for drug discovery are the following. Isolates of *C. albicans* from symptomatic patients with vaginal and oral candidiasis were significally more proteolytic than in asymptomatic carriers (*Cassone et al.* 1987; *De Bernardis et al.* 1990; *Agatensi et al* 1991; *De Bernardis et al.* 1992; *Wu et al. 1996)*. Strains of *C. albicans* with deleted Sap1, Sap2 or Sap3 genes caused less tissue damage in the *in vitro* RHE model (*Schaller et al.* 1999), less adherence to buccal epithelial cells (*Watts et al.* 1998) and less virulence in a rat vaginitis model. Also, Sap2-deleted strains were almost avirulent in the rat vaginitis model (*De Bernardis* 1999). The degradation of host tissue by Sap1-3 seems to be correlated with the ability to adhere to host-cell surfaces and cause colonisation. During mucosal infection Sap1-3 seems to be the most important of the Saps, and during deep systemic infections Sap4-6 seems to be the most important Saps (*Naglik et al.* 2003). Some evidence that Sap7-8 might be important during infections has also been reported (*Naglik et al.* 2003).

Protective effects have been seen upon pepstatin A treatment of *Candida* in murine and rat mucosal models (*De Bernardis et al.* 1997; *De Bernardis* 1999). Clearance of *Candida* infections in human patients is correlated with Sap inhibition in HIV-infected patients that receive HAART-PI (highly active anti retroviral therapy-protease inhibitors) (*Cassone et al.*1999,2002). They receive proteases inhibitors that are designed as drugs against the HIV-1 protease. Since these inhibitors also have some affinity for the similar Saps from *C. albicans*, (*Pichova et al* 2001) this affects the possibility for *Candida* to sustain oral infections. However, that HAART-PI treatment does not prevent oral candidiasis have also been published (*De Bernardis* 1999). Similarly, a renin inhibitor (A-70450) was found to be a potent inhibitor of Sap2 (K_i value of 0.17 nM). However the inhibitor gave no protection in the murine disseminated-infection model (*Abad-Zapatero et al.* 1996). With our current knowledge this is not so strange, since the murine disseminated-infection model is model for systemic disease, which correlated with Sap4-6 expression (*Stiab et al.* 2000) and we have no information about the inhibitory effect on these isoenzymes. Abbott has not shown work with other Saps and has not published any data from mucosal models.

This pinpoints the need to work with many different Saps. Also it shows that it is of great importance to test inhibitors in the appropriate infection model. Although Saps are indeed good drug targets, one should probably target both Sap1-3 and Sap4-6 if one wants to prevent all kinds of *C. albi-*

cans infections. Targeting Sap1-3 will be useful against mucosal infections and Sap4-6 against deep systemic infections. Inhibitors of Sap1-3 may be promising as topical drugs against mucosal *Candida* infection.

The Saps that I have focused my research on are Sap1-3. Saps are known to be produced by most, if not all, of the *Candida spp* that cause infections, so these targets could perhaps be used against all *Candida spp*. However the same questions of which Saps are associated with which type of infection must be further assessed in other *Candida spp*.

Biosensor technology and interaction kinetics

SPR-based biosensor technology, is based on the immobilization of a molecule to a dextran matrix, followed by injection of another molecule during continuous flow over the resulting sensor surface. The change in the surface plasmon resonance (SPR) angle over the surface versus time is measured and plotted in a sensorgram (Figure 7). This technique makes it possible to study interactions in real time.

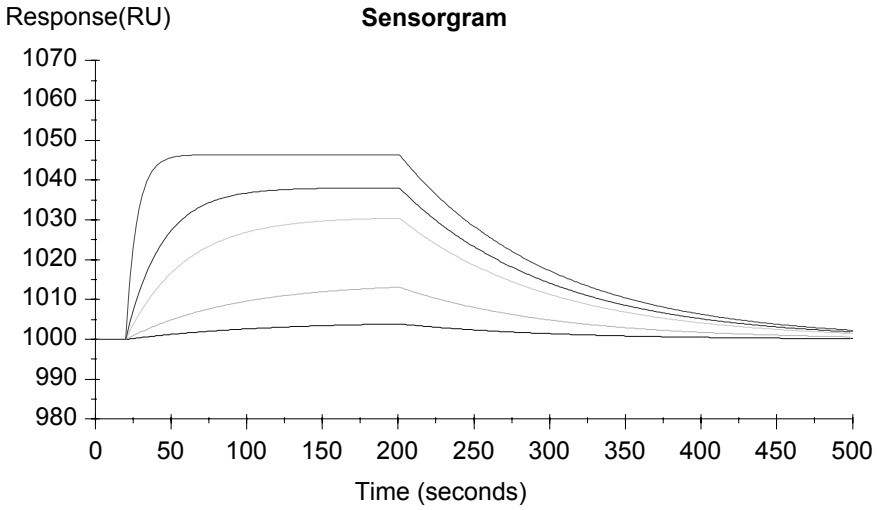

Figure 7. Typical concentration series of sensorgrams. Association is seen upon injection of analyte (start at 20s), dissociation is observed when injection ends (at 200s).

Fitting a suitable interaction model to a series of sensorgrams derived at different concentrations (Figure 7) makes it possible to determine the association and dissociation rates (k_{on} and k_{off}) as well as the equilibrium disso ciation constant of the complex ($K_D = k_{off}/k_{on}$). K_D gives a measurement of the affinity for the interaction between enzyme and inhibitor. There is no need for substrates during biosensor studies, so only one equilibrium is studied (Figure 3).

Although affinities are typically used to characterize interactions, information about the individual rate constants provide additional information. Association rates are more dependent on long-range interaction (eg. electrostatic interactions) (*Shen et al.* 1996; *Baerga-Ortiz el al.* 2000) compared to dissociation rates, that are more dependent on short-range interaction (eg. hydrophobic effects and or van der Waals interactions. As the same K_D can be obtained from different combinations of association and dissociation rate constants (*Markgren et al*. 2000), it is essential to understand which of these parameters is best correlated with *in vivo* efficacy. By understanding what forces are of importance for a certain interaction it can thus be possible to design inhibitors with optimal characteristics.

The interaction between two molecules can be illustrated by a simple reaction scheme (Figure 8).

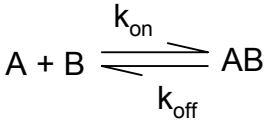

Figure 8. A simple interaction scheme for two interacting molecules. In biosensor-based technology B is the immobilized molecule and A the injected molecule.

This model can be described by the 1:1 binding model, mathematically expressed by three different equations (Eqs.1-3) for the association phase and by two equations (Eqs.4-5) for the dissociation phase:

Association

Eq1. $B(t)= R_{max}-R(t)$

Eq2. $dR/dt= - (k_{on}* C * B – k_{off} * R(t))$

Dissociation

Eq3. $R(0)= 0$

Eq4. $dR/dt= -k_{off} * R(t)$

Eq5. $R(0)= R_{d0}$

Where R(t) is the response at time t, corresponding to the concentration of AB, A = C (the concentration of analyte and it is constant), $B(0) = R_{max}$ (the concentration of immobilized molecule is the same as the maximum response), R_{d0} is the response at the start of the dissociation and $dB/dt = -(k_{on}*A*B – k_{off}*AB)$.

Inhibitors that exhibit very fast association will show mass transport limitations. Mass transport is the capacity of the buffer flow and diffusion to transport the analyte to and from the surface. Limited mass transport can be detected as effects on the estimated association rate by variation in the flow rate. In such cases, a factor compensating for limited mass transport can be

added to the 1:1 model. The Gibbs free energy of interaction from equilibrium constants was calcutated from: $\Delta G° = RT\ln K_D$.

Kinetic constants and their dependence on pH

Interactions between molecules are influenced by pH and can be to cause differences in the kinetic constants. It is therefore important not to compare, for example k_{cat}/K_M, K_i or K_D when the measurements have been performed at different pH values.

Another aspect to consider is that interactions should be studied under physiologically relevant conditions, In the case of Saps, these conditions may vary, so meausurments should be performed at several pH-values. This is facilitated by using the biosensor-based method, as a substrate-based method will have a more complex pH dependence due to effects on the enzyme-substrate interaction and catalysis.

Administration of protease inhibitor drugs against candidiasis

In drug development drugs are aimed to be potent enough to be administrated orally, simplifying the medication. Patients that need to take drugs each day or several times a day should not have to go to a hospital to get their drugs. As oral drugs work inside the body on some specific cell or against a virus that lives inside cells, the administration and distribution, of the drug is critical for *in vivo* efficacy. In order to compensate for poor adsorption, distribution, metabolism and excretion and for high toxicity (ADME-Tox) the drug should have very high *in vitro* efficacy.

In treatment of mucosal candidiasis (oral and vaginal) another possibility arises. The fungal pathogen is localized to the site of infection and drugs targeted against secreted aspartic proteases need to be active in the oral or vaginal cavity. It should therefore be possible to use topical administration of the drug. This would clearly alter the requirement for very high affinity inhibitors.

In general, competitive inhibitor drugs against enzyme targets are considered to need sub-nanomolar or at least nanomolar affinities in order to be potent enough upon oral administration. In the case of topical administration we really do not know what magnitude of affinity is needed. But since administration, distribution and metabolism of a topical drug is a lot simpler during local fungal infections, it is reasonable to assume that it should be lower than with oral administration. However, high affinities are generally always desired, since specificity is often achieved if the interaction is optimised for the target.

Present investigation

Aims

To study the fungal pathogen *C. albicans* and its use of secreted aspartic proteases (Saps) as virulence factors. The specific aims of the current project have been to produce these enzymes in order to develop methods that could be used to characterize Saps as targets for drug development.

Experimental strategy

1. To produce and purify active drug targets (Sap1-3)
2. To develop assays for activity, inhibition and interaction studies
3. Characterization of substrate and enzyme specificity of Sap1-3
4. Characterization of transition-state analogue inhibitor interactions with Saps 1-3

Experimental methodology

Cloning, expression and purification

In order to perform detailed experimental studies of an enzyme it is necessary to have enough of the enzyme in a reasonably pure form. The three different Saps were obtained by three different methods. The method for Sap1 and Sap2 started with obtaining a clinical isolate of *C. albicans*. In the case of Sap1, total genomic DNA was isolated and the gene for Sap1 was amplified with PCR technology. It was purified, digested and ligated into a plasmid that was transformed into *Escherichia coli*. Plasmid DNA was purified from *E. coli* and sequenced to control that the correct gene had been amplified and that the PCR had not altered the genetic code of the gene. The gene was then sub-cloned into a shuttle vector that was inserted into the fungi *Pichia pastoris*. The gene was overexpressed and the Saps became secreted into the medium by adding 1% methanol to the growth medium.

In the case of Sap2 it was possible to use knowledge about what triggers expression *in vivo*. Growth of *C. albicans* in a medium containing bovine serum albumin (BSA) as the sole nitrogen source forces *C. albicans* to induce expression of Sap2 with subsequent secretion of the enzyme into the medium where Sap2 degrades BSA. By this method, *C. albicans* can then import amino acids and dipeptides that can be utilized as nutrients and used in its nitrogen metabolism.

Sometimes talking to colleagues will save you time. A *P. pastoris* Sap3 (*Borg-von Zepelin et al.* 1998) expressing strain, similar to the one described for Sap1, was received from professor Michel Monod (Centre Hospitalier Universitaire, Lausanne, Switzerland) whom I met at two conferences. Interacting with colleagues will increase the quality and quantity of the scientific work that is done.

In all three cases, the expressed Sap was secreted into the medium. After harvesting of *P. pastoris* or *C. albicans* culture Saps were either precipitated or bound to a batch anion exchange gel, then dissolved or eluted in a smaller volume of buffer. The small volume of buffer (containing Saps) was then subjected to a column anion exchange chromatography step, which yielded reasonably pure (single band on a Coomassie stained gel) Saps.

To be able to use the original species for expression of the protein that one would like to study must be considered to be fairly unusual, but in this case it works. One clear advantage is that it will be the same protein as *in vivo*, and in addition, one does not have to clone the gene (which can be time consuming). A possible drawback is that one can have problems with purification due to expression of homologous proteins, which can be difficult to separate from each other. However, no such problems were encountered in this project.

The *Pichia* expression system for Sap1 and Sap3 worked well and gave good yields (30-60 mg Saps/L. culture). Since the gene is inserted in the genome of the fungi, the transformed *P. pastoris* strain was stable compared to the *E. coli* expression systems. Another advantage of the *Pichia* expression system is that it is a eukaryotic system that performs posttranslational modifications not done by *E. coli* expression systems. It was also possible to let the fungi secrete the Saps in a similar way as is done when they are expressed *in vivo*. Some drawbacks of the *Pichia* expression system are the higher cost and that it is more time consuming compared to the *E. coli* expression. However, it is much cheaper than other eukaryotic systems, such as human cell lines, and gives a much higher expression level in a shorter time.

In choosing the right expression system it is most important to consider that the expressed protein must resemble the protein expressed *in vivo* as much as possible. Proteins that are processed after expression will have different physical and chemical properties that can change catalytic activity or inhibitor binding. In a drug discovery project this is very important, and in the worst case one might develop drugs that will not work *in vivo*. So it feels comfortable to know that the enzymes that I used in my research closely resemble the protein expressed *in vivo*. Another aspect that is important to take into consideration is the expression level. High expression levels often facilitate purification of the protein and will not be a limiting factor regarding what one is able to study.

Take home message: **Work with targets which resembles the *in vivo* target and be sociable.**

Enzyme activity measurements

The catalytic activity of Saps was studied by following the hydrolysis of octa-peptide substrates that have para-nitrophenylalanine in the P1' position. This residue allows the rate of hydrolysis to be followed spectrophotometrically at 300 nm (*Dunn et al.* 1984; *Fusek et al.*1994). Activity measurements in 100 mM acetate buffer at pH, 3.8, 4.4 or 5.0 supplemented with 150 mM NaCl were performed. Substrate was equilibrated in buffer and the reaction was started by addition of enzyme. Alternatively, enzyme was equilibrated in buffer and the reaction was started by addition of substrate. All of the expressed and purified Saps were active and were inhibited by a general aspartic protease inhibitor, acetyl pepstatin.

These proteases are expressed and secreted during infection at different locations in the body. The different environments that exists at these local infection sites is highly relevant for a drug discovery project. Activity was studied at different pH. In order to elucidate whether the lower activity at

higher pH is due to a slower reaction (lower k_{cat}) or a decreased affinity for the substrate (higher K_M). Due to limited solubility of the substrates, it was often not possible to determine k_{cat} and K_M values individually instead the k_{cat}/K_M could be determined (Figure 10). Also, the substrates used during these studies were not very sensitive, due to low signal levels and high noise levels. Because of the low sensitivity and the need for inhibition studies at sub-optimal pH, other methods had to be developed.

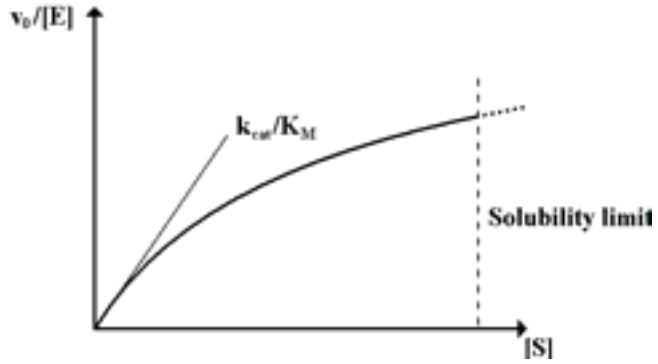

Figure 10. The case often seen for Saps where limited solubility of the substrates prevent K_M determination.

Substrate specificity, library design and assay

Substrate specificity of proteases cannot be determined one position at a time as the identity of a residue in a certain position may influence what can be accepted in another position, i.e. there is a subsite adaptivity. Therefore, libraries with two wobbled positions were synthesized by solid phase synthesis. This approach also facilitateted the synthesis of these substrates libraries. Synthesis was performed with the twenty amino acids naturally occurring in proteins, except for cysteine which was excluded and methionine, which was replaced by norleucine.

The design of these libraries started with the peptide KPLEF*nFRL, where * indicates the bond that is cleaved and nF is p-nitrophenylalanine. The P1 library generally had the sequence: K P Z E P1*nF X L. Where X is the varied position and Z is a wobbled in order to allow subsites to adapt to the residue in X. For each new P1 position 19 new pools are generated (with 361 different peptides for each P1 position). In the second case also the Z position can be studied. However in this thesis X describes the position that is studied and Z the wobbled position. Both positions should ideally consist of an equal mixture of 19 different amino acids. See Paper I for a more detailed description of the libraries.

The substrate specificity was determined by measuring the activity for a pool of 19 different peptides at a time. The data was normalized with respect to the most active peptide pool in each library. In the same manner the substrate specificity for all the six positions P3-P3' were investigated.

A clear advantage of using substrate specificity instead of inhibitor specificity to evaluate subsite specificity is that the peptide is certain to actually binds in a productive manner, otherwise no change in absorbance can be measured. It is not possible to use a kinetic assay to see if an inhibitor binds in the predicted orientation, so inhibitor interactions are less reliable for subsite definition.

Inhibitor library

The transition-state inhibitors studied in this project were originally designed for HIV-1 protease (*Alterman et al.* 1998; 1999) and their interactions with this protease were characterized (*Markgren et al.* 2002; *Gossas et al.* 2004). Structures of some of these inhibitors is shown later in this thesis. Furthermore, six clinically used inhibitors were characterized for interaction with Sap1-3. The aspartic protease inhibitors acetyl pepstatin and pepstatin A were used to evaluate purified Saps, binding levels and for assay design.

Biosensor assay for Sap1, Sap2 and Sap3

The possibility of setting up a biosensor-based interaction assay for Saps was explored as the activity-based assay had several severe limitations. The new assay was developed along the same principles used for setting up an assay for HIV-1 protease but with the exception that Saps generally are more sensitive to neutral and basic environments. However the low pI values of Saps (4-5 for Sap1-3) were not compatible with the amine coupling method. This method requires a pH at least one pH unit below the pI of the protein is used, whereby the protein is preconcentrated to the dextran surface. However, the amine coupling reaction requires that the pH is not acidic (*Johnsson et al.* 1991).

Therefore amine coupling could not be used and Saps were instead biotinylated and bound to streptavidin coated chips. This resulted in relatively stable surfaces with 4-8 kRu of immobilized Sap. Although baseline drifts were evident, they could be corrected for in the analysis.

Activity of biotinylated Saps and interaction between acetyl pepstatin and immobilized Saps

Assay development is a very important step in biochemistry. If one does not take this seriously there is a great risk that all work will be done for nothing

or even worse, might mislead other people that work with other scientific problems. If the properties of the proteins are altered by some kind of tag it is important to make sure that this does not change the activity of the enzyme or the affinity for ligands and one should always bear in mind that the tagged protein is not exactly the same entity as the physiologically correct one.

Due to the fact that the target has to be immobilized, in the biosensor assay, one must always check that the immobilized ligand behaves in a similar way as the soluble form. Therefore the activity of biotinylated Sap1-3 was evaluated. Since similar activity was obtained (75-95% of the activity was retained upon biotinylation and freezing and thawing of Sap1-3) it was concluded that the biotinylation did not significantly affect the function of the enzymes. In addition, acetyl pepstatin was used to evaluate the binding to Sap1-3 sensor surfaces. The K_D values obtaind with the biosensor method were compared with K_i values calculated from IC_{50} determinations and with literature values. From these comparisons it was possible to conclude that the K_D values were similar and followed the same trend (higher affinity for Sap2 than for Sap1) as the K_i values. Furthermore the K_i and K_D values determined for Sap1 and Sap2 were of the same magnitude as previously published (*Koelsch et al.* 2000). So the Saps sensor surfaces had the expected characteristics of functional enzymes.

Evaluation of activity/inhibition and biosensor data

In order to derive kinetic and equilibrium constants, such as K_D, k_{on}, k_{off}, k_{cat} K_M and K_i, from experimental data, one needs to fit equations to the data received from measurements. It is of great importance to use models that really describe the experimental data. This has been obvious throughout this whole project.

Firstly, we consider the enzymatic assays that have been used. In general, the assay was not sufficiently sensitive for many of the Sap-substrate combinations, so k_{cat} and K_M values could not be determined due to limitation in solubility and/or too high absorbance, causing deviations from linearity of the Lambert-Beers law that render the data not reliable. Not only does this mean that it will not be possible to determine whether changes in activity (k_{cat}/K_M) are observed because of a change in binding affinity (K_M) for substrates or due to a slower catalytic reaction step (k_{cat}). It also prevents reliable analysis of whether the Saps really follow Henri-Michaelis-Menten kinetics. Despite this, it was possible to determine the specificity constant (k_{cat}/K_M), but if the enzyme does not follow Henri-Michaelis-Menten kinetics these values will not reflect true specificity constants. It is therefore essential to have sensitive and accurate assays that allow clear distinction between different models.

When analysing inhibition expiriments by fitting equations for competitive inhibition to the data one should be aware that true K_i values can only be derived if one uses the correct model. Furthermore, if the sensitivity of the activity assay is low it will be even worse when measuring the reduction of enzymatic activity (hydrolysis, in this case) as is done during inhibition studies. Because I was interested in the altered affinities for inhibitors due to changes in pH, the situation became even worse since in normal cases inhibition studies are performed at optimal pH for catalytic activity. In this case inhibition would have to be performed at sub-optimal pH, which was not possible to do with enough accuracy.

Moving on to the biosensor measurements, this assay clearly has a higher sensitivity compared to the inhibition assay. It is a much simpler system to study (as described earlier in the introduction, Figure 3). A number of different models have been used to analyse biosensor data. Generally inhibitor interaction with Sap1-3 follows the simple 1:1 binding model.

Inhibitors that exhibit very fast association will show mass transport limitations. When this has been the case; a factor compensating for limited mass transport has been added to the 1:1 model. The evaluation of mass transport limitation is important since the sensorgrams for interactions with extremely fast association will not contain any rate information at all (completely transport limited interactions) (*Karlsson et al.* 1999; *Markgren et al.* 2001). In addition, high flow rate and low immobilization levels have been used to minimize mass transport limitations.

In all cases, a limited amount of baseline drift has been evident. This has also been taken into account by inserting a factor for baseline drift. The drift was determined manually for every injection. When the affinity of the injected inhibitor was high some evidence that the drift was analyte-dependent was seen. Therefore, a model that compensate for analyte-dependent baseline drift (*Markgren et al.* 2001) was used. Analyte-dependent baseline drift is when the drift is dependent on the amount of bound analyte. This implies that the activity of the Saps somehow causes the baseline drift. Degradation of streptavidin or self-degradation by Saps was not seen, so no conclusive evidence for this model was found. However, the fit (when using the analyte-dependent baseline drift model) was better and the sensorgrams showed no baseline drift at saturated binding, so this model was chosen.

The differences in the kinetic constants, between the data obtained with the model with the factor for analyte dependent baseline drift and the model with the factor for normal baseline drift were small, (about 5% for association and dissociation rate constants and 10% for the affinity) and affected the constants in a similar ways in all analyses. Therefore none of the conclusions drawn in this study would be affected if, in fact, the analyte-dependent model were inaccurately used.

Results and discussion

Substrate and enzyme specificity

For the development of Sap-inhibitors as anti-*Candida* drugs it is of importance to study and learn as much as possible also about potential substrates that can be hydrolysed by these enzymes. As *Candida* needs to degrade many different tissues, there are several physiologically relevant substrates. However, it is of interest to determine how the set of Saps together has the overall required substrate specificity and how the different isoenzymes contribute to this specificity. Also, this fungal pathogen can infect a number of animals. These other hosts have slight differences in protein sequence compared to the human proteins. By addressing substrate and enzyme specificity one can start to draw a picture of what might be hydrolysed by different Saps.

Also, some of the Saps are expressed during the yeast growth and others are expressed during hyphal growth. Hyphal growth mainly occurs at pH 6-7 and is associated with expression of Sap 4-6, isoenzymes with relative high pH optimum. Sap1-3 however seems to be dominantly expressed during the yeast form of growth. The need for different Saps as a result of altered growth patterns seems to exist. So the specificity of both substrates and isoenzymes is relevant to investigate in order to understand the *in vivo* functions of Saps.

An even more important contribution that can come from specificity studies is that one gains knowledge about which amino acid residues that can be accepted in different subsites. This information can be used when one tries to design inhibitors against the Saps.

In order to obtain this type of knowledge I have collaborated with professor Ben Dunn at the University of Florida, Gainesville, FL, USA. Due to the fact that I have done some evaluation of the data from this substrate specificity paper (Paper I), some aspects not included in the paper will be presented here.

Subsite specificity for Sap1 and Sap2

The substrate specificity for P3, P2, P1, P1', P2' and P3' was investigated for Sap1 and Sap2 (Paper I). In the P1 position, both Sap1 and Sap2 accepts hydrophobic residues, such as phenylalanine, leucine and norleucine. This is typical of aspartic proteases. However, the fact that Sap2 also can accept the basic residue lysine has not been observed for other aspartic proteases (Figure 11).

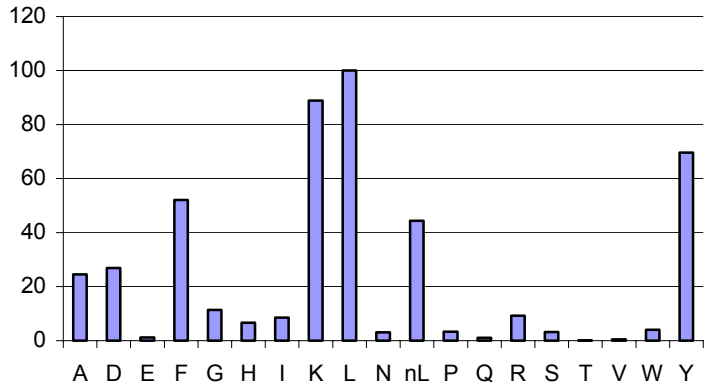

Figure 11. P1 position for Sap2, leucine and lysine is the best accepted residues for Sap2 in the P1 position. Results from the peptide KPEXF*nFLZ pools.

The peptide KPXEP1*nFZL pools, where the P1 position was phenylalanine, leucine, norleucine or tyrosine, was used to investigate the P3 position specificity. Sap2 prefers the hydrophobic amino acid residues in the P3 position. F, I, L, nL, V and Y were accepted for all different P1 residues. W was also accepted in the P3 position for all P1 residues except when P1 residue was norleucine. This is an example of the subsite adaptivity that was expected. So if the P1 position is occupied with a norleucine then tryptophan is not as well accepted in the P3 position. In general, substrates with glycine, histidine, lysine and arginine in the P3 position gave low activities.

The same investigation performed with Sap1 showed about the same relative acceptance. Sap1 prefers hydrophobic residues in the P3. Generally, in the P2 position Sap2 prefer I, E, L, nL and S. however, Sap1 preferred only isoleucine. For a schematic view of the preferred and not accepted residues see Table 1.

Enzyme	P3	P2	P1	P1’	P2’	P3’
P Sap1	FILnLVY	I	FLnL	EYW	KRTVY	I
N Sap1	KRHNSQAGTDP	KHRNQAGTFYLFW	GAVINSRDE	nd	GPDW	KGDP
P Sap2	FILnLVY(W)	IELnLS	FlnLK	EDInYV	V	IL
N Sap2	KRHNSQAGTEDP	GP	GINPQRSTVWEH	nd	DEGP	nd

Table 1. Relative acceptance for different sub-sites in Sap1 and Sap2. P means preferred (over 50% activity), N means not accepted (less then 15% activity), nd means not determined or no conclusive results. This table should be regarded as a general preference and not acceptance table and will not be valid for all combination of peptides.

So from these data it was possible to predict that, for example, Sap1 will show have high activity for the peptide KPFIL*nFRI. Furthermore, Sap2 should show rather high activity for the peptide KPFIL*nFKL. Although the Sap1 substrate has not been tested yet, the Sap2 substrate was characterized (Paper II) and revealed a k_{cat}/K_M value of about 7.3 $*10^5$ $M^{-1}s^{-1}$ and a K_M value of about 60 μM.

Subsite adaptivity

When lysine is placed in the P1 position instead of phenylalanine (Figure12), the substrate specificity of Sap2 is altered so that the enzyme prefers glutamate and isoleucine at P3 instead of isoleucine and leucine and also, arginine and other basic residues at P2' instead of valine.

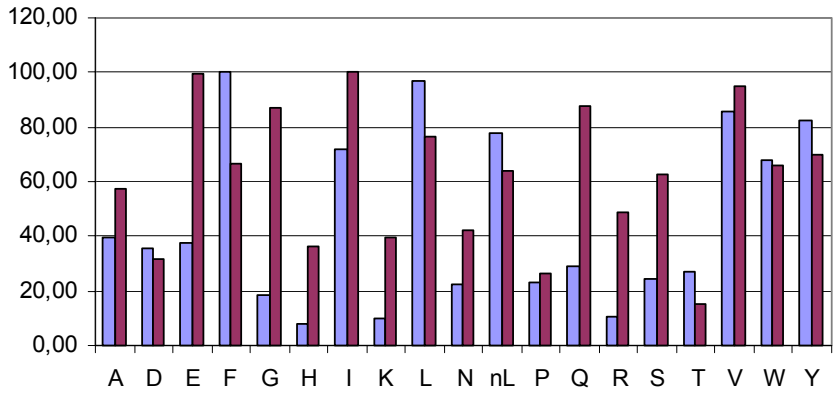

Figure 12. P3 specificity as a function of P1 position. Subsite adaptivity, Sap2 with two peptide pools. First bar KPXEF*nFZL and the second bar KPXEK*nFZL.

The substrate specificity studies that were done during this project showed clearly that different subsites in the protease-substrate complex were able to affect other subsites. This can be interpreted to mean that the subsite adaptivity in the enzymes allow adjustments of the structure so that it becomes possible to fit different amino acid residues into the different sub-sites depending on what residues are fitted to another subsite. To simplify one can say that what is fitted to one position will affect what can be fitted to the other positions. This is nothing new in general. It is a well-known fact that enzymes bind substrates with a so-called induced fit. However, this shows that one should not, as often is done, investigate substrate specificity to find optimal substrates by optimizing one position at a time. This should also be thought of when trying to design inhibitors against Saps. A more randomized approach may give some results that could not have been anticipated.

Method problems and interpretation of specificity results

Indeed, there are some problems with this type of library approach. The substrate concentration is about 100 μM during these experiments, which corresponds to about 5,25 μM of each substrate. Initial velocities are used for comparisons. In general, one would use k_{cat}/K_M values to compare substrates. During conditions of substrate concentrations that are very much lower than K_M the initial velocity is proportional to k_{cat}/K_M, which would mean that none of the peptides should have K_M values below 50 μM. This is not the case (since some of these substrates have K_M values as low as 10-20 μM), so peptides that have low K_M values will show lower activities than they actually have.

Furthermore, some substrates might have a limited solubility, which could cause them to precipitate in the stock solution. However, because the measurements are done at low concentrations, the stock solution should not have to have a higher concentration than about 65 μM. Very few, if any, of these peptides will precipitate at those concentrations. Another thing that is relevant to consider is that one could not be certain that the wobbled position is equally distributed. Although the reactions were done in order to get equal distribution there might be some fluctuation.

It should also be noted that all results from this study were normalized to 100% for the most active residue per library. This means that if one library has a lot higher activity than another library, then even a low acceptance (low % cleavage) from that library could show a relatively high activity. So one must consider this to be relative acceptance. Also, if one residue is only accepted 25% as well as the best this is just a four fold lower activity. Standard deviations during these measurements were as high as ± 20%, and this should be taken into consideration when using these results.

If one considers all of these problems one might conclude that it would be better to do the measurements on pure single peptide substrates. This would be great, but is there any lab that would do that study? Consider that if 19 different amino acids should be tested in six different positions then you would end up with 47 million different substrates. Sometimes one has to go for quantity instead of quality. One has to choose between much information on a limited amount of substrates or some information about a great number of substrates. The important aspect is to not over-interpret the results.

Substrate specificity information for inhibitor design

In this paper (Paper I) high affinity inhibitors (K_i= 3-29 nM) for Saps designed from substrate specificity data are presented. The design of these inhibitors were primarily based on the finding that Sap2 accepts lysine in the P1 position.

In general, aspartic proteases do not accept lysine in the P1 position. Therefore, these inhibitors do show high specificity for Sap2 compared with

human Cathepsin D. However, these inhibitors are useless as drugs since peptides have poor absorption and are generally degraded by human proteases. Furthermore, these peptides are octa-peptides with molecular masses around 1000, which is far too big for a good drug. But they still give a great amount of information on what residues the Saps can accept in different positions. Also, the affinity for those types of inhibitors suggests that this could be useful as a drug design approach. As noted earlier the design of inhibitor should probably be done rather randomized, not generalizing about what might be accepted, it is better to actually try what fits.

However, if one likes to be a bit critical, that is actually the way one does drug design today: testing a great number of different inhibitors that might work, just to find that some work and others do not work, and then we call it rational design. I would like to call it semi rational design.

Specificity constants for pure substrates with Sap1-3

In order to control whether the results from substrate specificity studies using pools (Paper I), are actually valid, eleven different substrates were assayed for activity with Sap1-2. Sap3 was also included during this study (Paper II). The results from measurement of single substrate support the subsite adaptivity described in paper I.

It was also found that the specificity constants for Sap2 were higher than for Sap1 and Sap3 for many substrates, even though their relative acceptances might be similar. Therefore, it is important to understand that normalizing results for each library can give misleading information when comparing the acceptance of amino acid residues by the different Saps. Consequently, the normalized values should not be used to compare the activity between different Saps for a particular amino acid in some subsite. They should be used only to compare the relative acceptance of different residues, as noted earlier.

When trying to draw conclusions about which substrates might be used *in vivo*, it is important to use the specificity constants. However, these kinds of analyses are complicated by the fact that enzyme concentration *in vivo* can be high and the local substrate concentration rather low. These circumstances are very different from the *in vitro* conditions.

All of the substrates that were soluble enough to be used in the assay showed activity and were possible to use for studies of Sap 1-3. This indicates that the information from the substrate-specificity study is relevant in the design of suitable substrates.

pH profile of activity

The activities of Sap1-3 have been studied at two different pH values (3.8 and 5.0) with a total of four different substrates. In all substrate-Sap combinations the sub-optimal pH of 5.0 showed lower specific activity. The differ-

ences in specificity constants due to the pH change between pH 3.8 and pH 5.0 were dependent on both enzyme and substrate.

This indicates that the activity of the Saps for many of the different *in vivo* substrates will probably have different pH dependencies. Since all *in vivo* substrates are not known and the exact pH at a certain infection site are not always the same, it will be impossible to calculate vitality values that actually can describe the physiological correct and very complex *in vivo* situation.

Biosensor interaction studies

HIV inhibitor screen

The approach to use a library of HIV-1 protease transition state analogues to find structures that could be used for further development into drugs against *Candida* infections is relevant, the compound with the highest affinity toward Sap2 known today is developed against renin. The synthesis of these compounds has already been worked out and this facilitates the development of analogues of these compounds. The screening of 104 HIV-1 inhibitors resulted in thirteen compounds that showed reasonable affinities and concentration dependent binding (Paper IV). These thirteen different compounds were further characterized. Acetyl pepstatin was used to control the binding capacity of the surface and to select interacting compounds based on percentage of the acetyl pepstatin response. In general, it can be concluded that these control injections should be done as often as once for every ten inhibitors screened to ensure reliable results.

Dissociation and association rates of interaction between Sap1-3 and transition state inhibitors

One of the more interesting results about the kinetics of binding, that this project yielded is the conclusion that the structure of BEA-410 somehow is prevented from associating (very slow association) with Sap1 (Paper IV), but when it has bound the stability of the complex is rather strong. This is a typical case where information from association and dissociation rates gives us information that can be used in further development of these inhibitors. In the case of BEA-410 the affinity is low and it is not likely that normal inhibition measurements would have revealed it as being of interest and impossible to find it special (slow dissociation).

Another exciting aspect of rate constants for Sap interactions with inhibitors is that they do not show the same dependence of pH (Paper III). Acetyl pepstatin showed a decreased affinity for Sap1 and Sap2 at pH 5.0 compared to 3.8, which was found to be due to a slower association rate. However the dissociation rate also decreased, thus working in the opposite direction (increased affinity). This can be interpreted as that pH changes affect the inter-

actions (between acetyl pepstatin and Sap1-2) determining the association and dissociation rates in different ways (different mechanisms) under the conditions used for these experiments. This can be of interest in a drug discovery project where pH is relevant for drug affinity. Other studies of rate constants done in our laboratory on HIV-1 protease and similar inhibitors as have been used in these studies (Figure 13a and 13b) also show that by gaining information about these rates it is possible to understand how to increase affinity for inhibitors (*Shuman et al.* 2003; *Gossas et al.* 2004)

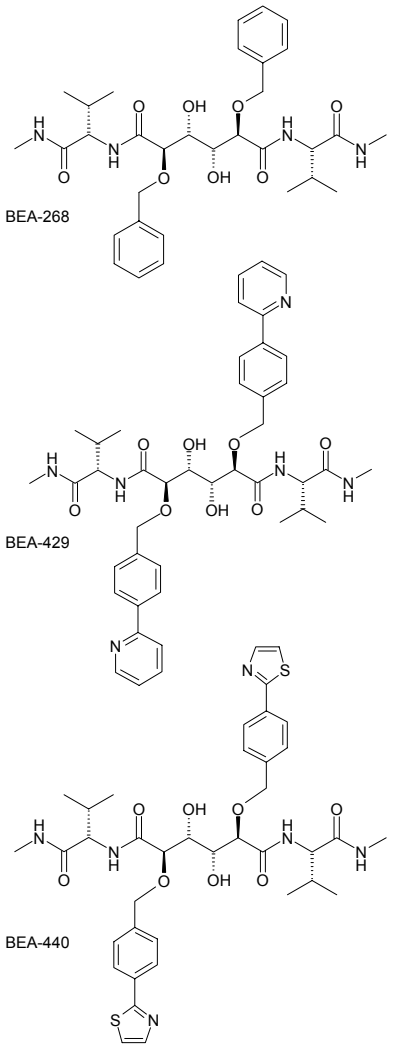

Figure 13a. Structures of Sap inhibitors, which has been characterized during this project

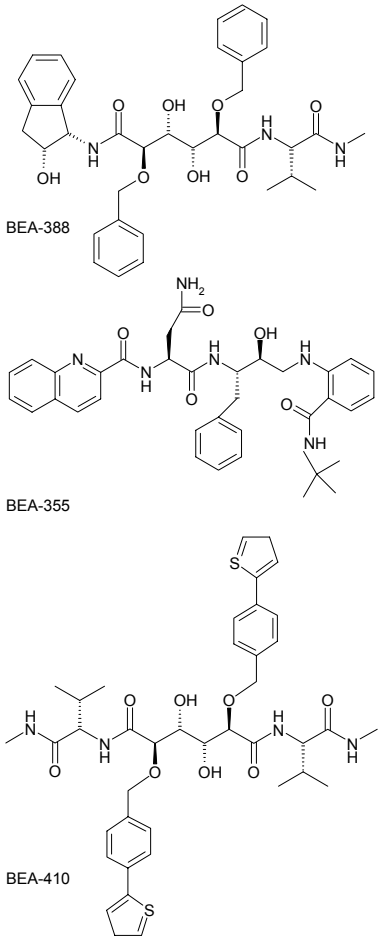

Figure 13b. Structures of Sap inhibitors, which has been characterized during this project

K_D, k_{on} and k_{off} for compounds with favourable kinetics

The equilibrium dissociation constants (K_D) for the compounds with favourable kinetics are, in most cases, 1-30 μM for Sap1-3. BEA-429 has the highest affinity (lowest K_D value) for Sap1 and Sap2 with about 1.5 μM for both. It has fairly fast association rates of about 1.7-1.8* 10^5 $M^{-1}s^{-1}$ and fast dissociation rates (0.28 s^{-1}). However, BEA-429s affinity for Sap3 is 18-fold lower compared to Sap1-2. This lower affinity for Sap3 is a result of both the association and dissociation rates for the interaction. Structural analogues of BEA-429, such as BEA-268 show about 28-35 times lower affinity for Sap1

and Sap2. This difference in K_D values for BEA-268 is only about two-fold higher for Sap3 compared to Sap1 and Sap2.

BEA-355 has about the same affinity for Sap1 as BEA-429, the difference in association and dissociation rate being about five fold. So BEA-355 showed a slower association, but compensates for this by a dissociation that is about five times slower than for BEA-429. For Sap2 the affinity of BEA-355 is about four-fold lower then for Sap1. Also, the affinity for Sap3 is about two-fold lower than for Sap1. The reason for Sap2s lower affinity is a four-fold faster dissociation rate compared to Sap1. BEA-388 has a K_D value of about 5 μM for Sap2. However, the K_D value, for Sap1 is 20 μM and for Sap3 over 100 μM.

A general trend among these inhibitors is that their dissociation rates are very fast. This is due to the fact that they have not been designed for Saps. In addition, the isoenzyme specificity for inhibitors varies, i.e. the affinities for a certain inhibitor varied for the different isoenzymes, and was unique for each inhibitor. There were no strong patterns although Sap1-2 were, in general, more similar to each other and Sap3 seems to be more different in its interaction behaviour with inhibitors. However, four-fold higher affinities for Sap2 compared to Sap1 have been observed. These conclusions pinpoint the need to study the inhibitor interactions for all relevant Saps.

The clinically used HIV-1 inhibitors

Six of the clinically used HIV-1 inhibitors were included in the screening (Paper IV). Of these inhibitors ritonavir showed the highest affinity. The K_D values for ritonavirs interaction with Sap1-3 were 0.5-4.1 μM. Ritonavir has the overall highest affinities for Sap 1-2 of all inhibitors that were assayed, except acetyl pepstatin and pepstatin A, which cannot be used as drugs. Indinavir and saquinavir showed K_D values of 25- >100 μM for the different Saps. Amprenavir showed slightly lower amount of binding in the screen compared to saquinavir and indinavir. Finally, nelfinavir and lopinavir (concentration of 15 μM) showed even lower amount of binding to the Saps in this study, which suggests that nelfinavir and lopinavir will not have any curative effect on mucosal candidiasis due to inhibition of Saps.

It seems likely that the clearance of oral candidiasis that has been reported is due to the inhibition of Saps by ritonavir. Doses of ritonavir are high but the bioavailability causes the local concentration of ritonavir in the oral cavity to be rather low. This maybe the reason for the incomplete clearance observed. The low affinities for Saps displayed by saquinavir and indinavir should not be enough to cause this clearance. Also the low amount of binding for amprenavir indicates that it would have the same problems as saquinavir and indinavir (low affinity).

pH profile for Sap1-3 binding of inhibitors

The pH profile of binding for Sap1-3 was studied due to the earlier mentioned relevance of the pH for binding to Saps during infection (Paper IV). All inhibitors that were studied showed a lower affinity at sub-optimal pH. The differences in equilibrium dissociation constants for compounds with preferable affinity were 2-6 kJ/mol. The decreased affinities should be expected, since the inhibitors are transition state analogues, and therefore should bind to the active site with the highest affinity during optimal catalytic conditions. The differences in decreased affinities that have been seen emphasize the relevance of studying the pH-dependences of binding, since different inhibitors are affected in different ways.

BEA-440 the interesting inhibitor

BEA-440 is my favourite Sap inhibitor identified during this project (Paper IV); the reason being that it is more complex than the other inhibitors in its binding properties. I postulate that the binding of BEA-440 induces a conformational change of the Saps upon binding. The reason for this conclusion is seen in the binding sensorgrams of BEA-440: it binds relatively slow and seems to have two different association phases.

Also, the dissociation phase is biphasic with two distinctly different dissociation rates. Injections with different injection times show that longer injection times give a higher degree of the phase with the slower dissociation rate. Inhibition measurements show higher apparent affinities due to pre-incubation of enzyme and inhibitor, which also indicate the existence of at least two different forms of complexes between enzyme and inhibitor.

There is actually another way to interpret the biosensor and inhibition data for BEA-440. If BEA-440 can bind to the active site in two different ways with different affinities, this would also explain the two-phased binding. This hypothesis can also explain the higher population of the slower dissociation phase during longer injection times, and also the higher inhibition seen upon longer pre-incubation between Sap1 and BEA-440 can indicate that an equilibrium between the two different binding sites are taking place.

It will not be possible to design an experiment of kinetic nature that can distinguish between these two different models, since they are represented by two identical equations. One might then consider that it does not matter which model is correct. However, in order to use the information to design new compounds we must know whether BEA-440 binds to the active site in two conformations or in two different modes. This would require access to the three dimensional structure of the apo-enzyme as well as the enzyme-inhibitor complex, preferably determined by a time resolved technique.

Further improvement of inhibitors and new design of inhibitors

In this part of the thesis I will speculate about further design based on existing data, so the basis for these speculations is scientific. However, the lack of inhibitor-Sap structures for these inhibitors and that it is today not possible to predict all of the interactions that can take place will limit these speculations. During this discussion I will be on thin ice.

The affinities for the compounds with favourable kinetics are 1-30 μM for Sap1-3. This affinity is far from acceptable for a drug with oral administration, but with further development of the structures they might become nM inhibitors. BEA-429 has the highest affinity for Sap1 and Sap2. It has a fairly fast association rate about ten-fold slower than, for example, atazinavir, saquinavir and about three-fold compared to nelfinavirs association rates for HIV-1 protease (*Shuman et al.* 2003). Dissociation rates are, however, clearly in need of improvement. About 100 times slower dissociation is found in the case of HIV-1 protease clinical inhibitors (*Shuman et al.* 2003).

Improvement of the affinity could maybe be focused on trying to improve the dissociation rate. One way to do this is to find van der Waals interactions and hydrophobic interactions that could prevent dissociation and increase the affinity for the compounds. This could be done by filling up the hydrophobic pockets in Saps sub-sites. Also, to find specific hydrogen bonds would be preferable. The interaction between Saps and BEA-440 has two different dissociation phases, of which one of these has a remarkably slow dissociation. However the association seems to be rather slow.

BEA-440 and BEA-429 might be further developed, and due to their differences and the fact that binding affinity comes greatly from the binding to S1 and S1' positions, as evidenced by the fact that BEA-268 (that only differ from BEA-429 in the P1 and P1' positions) has a clearly lower affinity for the Saps. There should be plenty of possibilities to develop these structures.

The difference in energies of binding between BEA-268 and BEA-429 is about 8-9 kJ/mol for Sap1 and Sap2. If one also could gain about 8-9 kJ/mol by exploring the P2 and P2' positions the K_D values for Sap1 and Sap2 would be in the range of 45-65 nM, which is about 10-fold higher affinity than ritonavir, that already shows some affect against *Candida* infection during oral administration of HAART-PI.

Furthermore, the structures of BEA-429 and BEA-440 are symmetrical, designed to fit the symmetry of HIV-1 protease. By abandoning the symmetrical structure one should gain further affinity, since the substrate specificity data indicates that the S1 pocket prefers less bulky residues compared to S1' sub-sites. This can be used if one synthesizes a compound that has the BEA-268 P1 residue and the BEA-429 P1' residue. Furthermore the substrate specificity studies indicate that lysine can be accepted in the S1 position. This could also be used for development of BEA-429 analogues.

BEA-440 and BEA-429 can be further developed in different ways since BEA-440 has a need for faster association, and BEA-429 would require a slower dissociation. One could try to find long-range interactions for BEA-440, whereas more work should be done to try to stabilize the complex for BEA-429. To be able to do this one would have to have a structure of the different Saps with a suitable inhibitor. However, this way of thinking, to optimise the dissociation and associations separately, is relatively new and I think that we need to understand more about what gives the fast association rates and slow dissociation rates before it can be used with success.

BEA-355 has about the same affinity for Sap1-3 (K_D values of 1.5-7 μM). This implies that the structure of BEA-355 fits all of these Saps, which can be of interest for the further design, since one inhibitor that block as many of the Saps as possible will be preferable. BEA-388 is structurally related to BEA-268. BEA-388 has higher affinity of about 2.5 kJ/mol for Sap1 and 5.1 kJ/mol for Sap2 compared to BEA-268. This could be used by, modifying the structure of BEA-429 with the P2 residue of BEA-388. This could give affinities that would be about 600 nM for Sap1 and about 200 nM for Sap2.

During this discussion affinities have been treated as if the were additive. Sometimes this is true, but in many cases reality is more complex than that. As has been presented earlier in this thesis, the sub-sites may communicate, so it is possible that one would lose affinity but also possible that one could gain affinity due to this adaptivity. Also, the interaction between BEA-440 and Sap1-2 needs to be better understood. It might be that BEA-440 does not bind to the S2S1S1'S2' sub site that it is designed for, or that it binds in the opposite direction. The synthesis of an inhibitor that has the BEA-268 residue in the P1 position, and the BEA-440 P1' residue, followed by characterization of that inhibitors interaction with Sap1-3, would be interesting.

Conclusions

Substrate specificity

The conclusion that can be made from the substrate specificity studies is that they give information about which substrates can be hydrolyzed by Sap1-3. Although this information can be utilized to find better substrates for enzymatic studies, it gives a very limited knowledge relevant for the *in vivo* situation. The reason for this is that Sap1-3, in general, has broad substrate specificity

Compounds

In this drug discovery project I have found five compounds that are candidates for further development. This development should use the information about association and dissociation rates generated during the biosensor stud-

ies of these compounds. Furthermore, the strategy to abandon the symmetrical structure will yield inhibitors with more relevant structures for Sap interactions. Also, the substrate specificity studies showed that there are differences between the acceptance of residues in the S2, S1, S1' and S2' subsites between the different Saps. However the sub site adaptivity implies that one should not try to design each subsite separatly.

Biosensor technology a flexible high information content assay

The biosensor assay has been very nice to work with. The fact that you only follow binding and are not limited to work under conditions optimal for activity combined with the much greater sensitivity makes it a better and more flexible assay than conventional inhibition measurements. Furthermore, one gains information about association and dissociation rates. The association and dissociation rates could, in the future, contribute to a better understanding of interactions.

X-ray crystallography

It is clear that the project needs to use structural methods to be able to understand the interaction between BEA-440 and Sap1-2. Inhibitors with reasonable affinities have been isolated and structural analysis of the enzyme-inhibitor complexes would help to clarify their interactions with Saps. It is possible to consider structure-activity relations due to the fact that some of the inhibitors are structural analogues. However, for further analysis the need for structural information about the enzyme-inhibitor complexes is evident. One way to go would be to dock the inhibitor to the structure that has been solved, but due to the rather low resolution and the subsite adaptivity, it is probably difficult to obtain useful data.

Who will be interested about *Candida albicans*

During this project I have meet many people who are not very interested in fungal disease. Also, candidiasis is often seen as a small problem, and not as a disease that actually kills people. Clearly candidiasis is not a very big problem if we compared it to HIV and malaria, which kills millions of people every year. In my eyes this fungal pathogen is to be taken seriously due to several factors. Firstly, decreased susceptibility and resistance has started to develop against drugs used against candidiasis. Multi-resistant bacteria are a huge problem today in medical care and we would not like to have multi-resistant fungal pathogens also. Secondly, these pathogens do attack people/patients that are already weakened from some other disease or surgery. Today we can prolong the lives of HIV-infected patients and cure many types of cancer, but with the risk of infecting these patients with candidiasis. More must be done to contribute to a better life for the patients that acquire these fungal pathogens.

Complex project

To target the Saps with transition-state inhibitors in order to design drugs against candidiasis is a complex project. The many different types of infections and the fact that there are ten Saps (of which six to eight seem to somehow correlate with some type of infection) complicate this project. However, many drug companies have large libraries of transition-state inhibitors against aspartic proteases. To use these libraries in biosensor-based screens for inhibitors against Saps are cost efficient, since the synthesis already has been worked out. Whether drugs based on Sap inhibitors will work remains to be seen, but it looks promising today.

Future work

Further studies of BEA-440

BEA-440 interacts with Sap1 and Sap2 in a mode that indicates the possibility of a conformational change this should be further characterized, perhaps by biosensor studies using concentration series with longer injection times combined with more inhibition measurements with more different preincubation times. A structure with BEA-440 would be preferable, since it should reveal interesting features about how the subsites can accommodate the functional groups.

Synthesis of new improved inhibitors

This work suggests some scaffolds that could be used for further synthesis. Data from the substrate specificity studies should also give some valuable information that should be tested.

Start to work with other saps (Sap4-8) and also work with Saps from other Candida species (*tropicalis and parapsilosis*)

C. albicans is not the only cause of candidiasis. There are many different species such as *C. tropicalis*, *C. parapsilosis* and *C. lusitaniae*. Although *C. albicans* is still the main cause of candidiasis, the increase of decreased susceptibility and resistance is mostly seen in some of the other spices. Also, to be able to treat systemic infections Sap4-6 should be targeted. I would suggest that the interaction between inhibitors and Sap1-8 should be studied and evaluated against *in vivo* data in order to understand the whole impact of Saps as virulence factors.

Further biosensor measurements

Interaction measurements of potential drugs could be done for many different Saps at a few different pH values, such as pH 3.8, 4.5 and 5.7 in acetate buffer and at pH 6.5 and 7.4 in phosphate buffer. Accumulation of as much

data as possible on how the potential drugs interact with different Saps will be crucial to an understanding of how these drugs really work and in which type of infections they can be used.

Challenge the dissociation constants, by rate constant and structural based design

It is clear that more information about enzyme-inhibitor interactions is gained by determining association and dissociation rates, which facilitates the interpretation of interactions. However, today no lab has yet reported any design based on association and dissociation rates. It may be possible to change this in the near future. Synthesis strategies should be based on association and dissociation rates. The aid of three-dimensional structure of Saps in complex with inhibitors will be crucial to the success of such a project.

Use all information for a fluorescence assay

If one would like to measure inhibition in order to do careful characterisations of potential drug leads it would be useful to design good fluorescence-energy transfer-based substrates. The sensitivity of these substrates is better and to be able to rely on inhibition measurements under sub-optimal pH, higher sensitivity would be needed.

Using biosensor and chromatographic assays for measuring other interactions of drug leads

Drug development is so much more than developing high affinity inhibitors of good drug targets. For a high affinity inhibitor to be a good drug one needs to evaluate ADME (Absorption, Delivery, Metabolism and Excretion) properties. Also toxicity must be considered. To obtain a good drug, there is still very much work to be done. The biosensor can be used to study interactions with some of the normally occurring transport proteins, such as human serum albumin and α_1-glycoprotein (*Cimitan et al* in press). But there are also other ways to do these studies, such as the chromatographic methods developed here at Uppsala University (*Beigi et al.* 1995; *Engvall et al.* 2004)

Cell assay testing

When drug leads have been improved they should of course be tested in some assay that is more relevant than *in vitro* biosensor assays. However, much analysis can be done before testing substances in animal (mouse or rat) models. A number of good models that mimic the animal models that might be suitable to minimize the number of animals that has to be used in the final tests. RHE model could for example be used to predict whether inhibitors will decrease the adherence of *Candida*. High affinity compounds that have good bioavailability and that show good efficacy in mimic models and are drug candidates must be tested in animal models in pre-clinical trials. We

should, however, strive to do this on as few and as good as possible drug candidates.

Clinical trial

Compounds that shows efficacy *in vitro* and potency *in vivo* and pass the pre clinical trial with toxicity tests should be tested in clinical trials both on patients that are infected by *Candida* and on people without infection. Drug potency and side-effects should be evaluated. This takes several years, which means that it will probably take at least ten to fifteen years before we see any compounds developed from this project in the pharmacy.

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Summary in Swedish

Candida albicans

I början av 1980 talet så började HIV viruset spridas över världen och i dagsläget har har miljontals människor världen över blivit smittade. HIV viruset attackerar immunförsvaret och orsakar ett tillstånd som benämns AIDS, i detta tillstånd har patienterna i stort sett inget immunförsvar kvar vilket gör att de är känsliga för alla slags infektioner. Inget vaccin har utvecklats trots, att enorma forskningsresurser lagts ner på detta.

Det finns heller inget botemedel mot HIV viruset, även om bromsmediciner har utvecklats som bromsar upp HIV virusets nedbrytningen av immunförsvaret. Det nedsatta immunförsvaret ger bakterier, andra virus men framför allt svampar möjligheten att infektera den redan lidande patienten. En av de vanligast svamparna som kan infekterar HIV-patienter är *Candida albicans*. I vanliga fall finns den på de flesta människor men orsakar ingen infektion, men under vissa betingelser startar den ett infektsiöst växt beteende.

Så i dagsläget finns det miljontals människor som bär viruset. Av dessa kommer 85-90% att få minst en infektion av *Candida albicans* och många av dessa får återkommande infektioner. Svampen infekterar dessutom friska människor och tre fjärde delar av den kvinnliga befolkningen kommer någongång att få erfara vaginal candidiasis.

Den ökande uppkomsten av *Candida* infektioner har medfört ökad användning av de befintliga läkemedlen med ökad resistens mot dessa läkemedel som följd. Så det redan begränsade antalet läkemedel mot candidiasis minskar som följd av denna resistens. Det är därför av största vikt att fokusera forskningen på nya anti svamp läkemedel.

Utsöndrade aspartat proteaser

Candida albicans utsöndrar aspartat proteaser (Sap enzymer) som är viktiga för svampens möjlighet att orsaka infektion. Att använda dessa enzymer som mål för läkemedel kan alltså var en intressant möjlighet. En del av de tidigare nämnda bromsmedicinerna mot AIDS är proteasinhibitorer och riktar sig mot ett viralt aspartat protease (HIV-1 proteaset). En del av de patienter som har fått proteasinhibitorer som läkemedel har visat tillfrisknande från oral candidiasis. Detta antas bero på att även Sap enzymerna inhiberas av HIV-1

protease hämmarna. Detta är inte orimligt då de båda enzymerna delar samma katalytiska mekanism och därmed har liknande trasition state. Hämmarna som är AIDS läkemedel är transition state analoger. Tillfrisknandet från oral candidiasis kunde också bero på en allmän förbättring av immunförsvaret hos patienterna, men det har visat sig att även patienter som ej fått bättre immunförsvar av behandlingen ökat sin motståndskraft mot oral candidiasis.

Forskningsmål

Min forskning har haft som slutgiltigt mål att forska fram proteas inhibitorer som kan användas som läkemedel mot *Candida albicans*. Ett delmål på vägen ditt är att identifiera molekyler som med viss affinitet binder till Sap enzymerna. Dessa molekyler strukturer kan sedan vara utgångspunkter för kommande design av bättre inhibitorer med läkemedel lika egenskaper.

Studier

I denna avhandling presenteras resultat från fyra studier. De fyra studierna kan delas upp i två olika delar. Den första delen tar upp substratspecificiteten hos Sap enzymerna och den andra tar upp Sap enzymernas interaktioner med inhibitorer. Den första delen har som syfte att undersöka vad som är möjligt för Sap enzymerna att katalysera. För att katalys skall ske måste enzymet interagera med substratet. Dessa studier ger alltså information om vad som enzymerna kan och inte kan interagera med.

I den andra delen fokuseras forskningen på enzymernas interaktion med hämmare. Där analyseras ett bibliotek av inhibitorer som är designade för HIV-1 proteaset. Denna del är fokuserad på att finna inhibitorer med viss affinitet. Dessa inhibitorer kan sedan utgöra en plattform för design av nya mer effektiva inhibitorer. Både enzymaktivitet och inhibitor-enzym interaktioner studerades också vid icke optimalt pH, för att efterlikna den skillnad som finns vid olika lokalisering av infektioner.

Modeller

Genomgående har det visat sig vara av stor vikt att man använder rätt ekvationer för att anpassning till laborativa data. Generellt har interaktioner mellan Saparna och inhibitorer kunnat beskrivas med en enkel 1:1 bindningsmodel. En av de inhibitorer som har visat affinitet för Sap enzymerna BEA-440 avslöjar dock en mycket mer komplex interaktion. Två distinkt olika dissociations faser är uppenbara. En förenklad modell för konformationsförändring ger den bästa anpassningen men är långt ifrån tillräckligt komplex.

Resultat

Substrat specificiteten för Sap1-3 har studerats och preferenser för hydrofoba aminosyror i P1, P2 och P2' positionerna har påvisats. En intressant upptäckt är att Sap2 accepterar basiska aminosyror såsom arginin och lysin i P1 positionen.

Vidare har substratspecificitetens adaptivitet påvisats, dvs vad som occuperar en bindningsyta påverkar vad som kan accepteras i de andra ytorna. Sap1-3s aktivitet under icke optimalt pH har också studerats. De olika substraten och de olika sap enzymerna visade olika pH beroende vilket betyder att pH är en viktig faktor att studera.

Fem stycken olika inhibitorer med viss affinitet för Sap1-3 har isolerats från ett inhibitorbibliotek. Deras strukturer ger information om hur man kan fortsätta designen av inhibitorer för att syntesera nya mer potenta inhibitorer. Informationen om substrat specificiteten bör också implementeras på inhibitor designen.

Slutsatser

Inhibitorer som kan vidare utvecklas med avseende på affinitet för Sap enzymernahar isolerats. Substrat- och inhibitor-studier har gett mycket information om hur man kan gå vidare med design av nya mer potenta inhibitorer. Studier av dessa föreningars interaktioner med biosensor teknologi kombinerat med röntgenkristallografiska strukturbestämningar av enzym-inhibitor komplex kommer att vara en effektiv väg mot potenta hämmare. Vägen fram till ett nytt läkemedel mot candidiasis är dock lång och därför är det av högsta vikt att denna typ av forskningen får mycket ekonomiskt stöd framöver.

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