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Yeast Growth Selection System for the Identification of Cell-Active Inhibitors of Human Cytomegalovirus Protease

THÈSE

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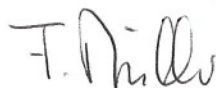
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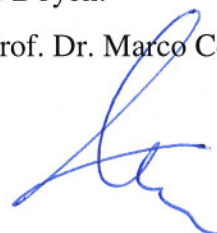
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TABLE OF CONTENTS

1.	Summary / Résumé	1 / 2
2.	Introduction	4
	2.1. <i>The cytomegalovirus</i>	4
	2.2. <i>Current antiviral treatments</i>	6
	2.3. <i>The cytomegalovirus protease as a target</i>	6
	2.4. <i>Biochemical versus cellular assays</i>	9
	2.5 <i>Conclusion</i>	10
	2.6 <i>References</i>	11
	2.7 <i>Figures</i>	15
3.	Results	19
	3.1. <i>Part I</i>	19
	A yeast growth selection system based on reporter gene activation to detect human cytomegalovirus protease activity (LexA-M system)	
	1. <i>Material and methods</i>	20
	2. <i>Results</i>	23
	3. <i>Discussion</i>	27
	4. <i>References</i>	29
	5. <i>Figures</i>	30
	3.2. <i>Part II</i>	34
	Novel yeast cell-based assay to screen for inhibitors of human cytomegalovirus protease in a high-throughput format (Trp1-M system) (paper)	

3.3.	<i>Part III</i>	42
	HTS and hit validation	
	1. <i>Material and methods</i>	43
	2. <i>Results</i>	49
	3. <i>Discussion</i>	58
	4. <i>References</i>	63
	5. <i>Figures</i>	66
4.	General conclusion	74
	<i>References</i>	78
5.	Appendix	79
	5.1. <i>Yeast plasmids and promoters</i>	79
	5.2. <i>List of abbreviations</i>	80
6.	Acknowledgements	81
7.	Attachments	82
	7.1. <i>Curriculum vitae</i>	82
	7.2. <i>Publications</i>	83
	7.3. <i>Patent</i>	83
	7.4. <i>Scientific conferences</i>	83

1. Summary

Human cytomegalovirus (HCMV) is one of the most common opportunistic infective agents in immunocompromised individuals, such as AIDS patients and organ transplant recipients, and causes the most frequent congenital infection in humans. Existing antiviral treatments are limited by severe drawbacks, i.e. poor bioavailability, toxicity and limited effectiveness mainly due to the development of drug resistant viruses. Thus, there is continuous need for new and effective anti-cytomegalovirus agents. The viral protease, which functions during assembly and maturation of the capsid and which is essential for the production of infectious virus, represents a promising drug target.

In Parts I and II of this PhD thesis I describe the development of two growth selection systems that monitor HCMV protease activity in yeast cells. In the LexA-M system (Part I) the cleavage site of HCMV protease is inserted between the DNA-binding domain of LexA and the activating domain of the transcription factor Gal4p. Cleavage by the viral protease inactivates the likewise engineered transcription factor, thereby causing a stop in transcription of a downstream cloned LexA-Gal4p regulated *HIS3* growth selection gene. Such yeast cells do not grow in selective medium lacking histidine. However, addition of a protease inhibitor allows expression of the *HIS3* gene resulting in stimulation of cell growth.

In the Trp1-M system (Part II), the HCMV protease cleavage site is inserted in Trp1p, a yeast protein essential for cell proliferation in medium lacking tryptophan. Functional inactivation of this modified Trp1p by the viral protease prevents cell growth, whereas addition of validated protease inhibitors results in stimulation of cell proliferation. Importantly, both systems rely on a positive read-out and therefore directly select for non-toxic compounds.

In Part III of this thesis, the LexA-M system was applied to a high-throughput screening (HTS) for the identification of HCMV protease inhibitors. A library of 15'000 small molecules was screened in a fully automated manner. We obtained 67 confirmed hits, which stimulated growth in yeast in a concentration-dependant manner. These 67 hits were further investigated in biochemical and mammalian cell-based assays. 8 of them showed good antiviral properties in cell culture in the 2-digit μM range; however no evidence was obtained for a direct binding to the protease.

1. Résumé

Le cytomégalo­virus est l'un des agents infectieux les plus communs chez les personnes immunodéficientes, tels que les malades du SIDA ou les receveurs de greffe. Ce virus cause aussi fréquemment des infections congénitales. Les traitements existants sont limités par des défauts majeurs: mauvaise absorption, toxicité, efficacité restreinte à cause du développement de souches virales résistantes aux médicaments. Il y a donc un évident besoin de nouvelles thérapies. La protéase du cytomégalo­virus, responsable de l'assemblage et de la maturation de la capside, est essentielle pour la production de virus infectieux, et représente donc une cible prometteuse pour le développement de nouveaux médicaments.

Dans les parties I et II de cette thèse, je décris le développement de deux systèmes de sélection qui mesurent l'activité de la protéase du cytomégalo­virus dans la levure. Dans les deux systèmes, l'activité de la protéase est reflétée par un ralentissement de la prolifération cellulaire. Dans le système LexA-M (Partie I), le site de clivage de la protéase est inséré entre le domaine de LexA se liant à l'ADN et le domaine activateur du facteur de transcription Gal4p. Le clivage par la protéase inactive ce facteur de transcription hybride, causant ainsi un arrêt de la transcription du gène rapporteur *HIS3*. En conséquence, ces cellules ne peuvent pas proliférer dans un milieu sélectif sans histidine. Cependant, l'ajout d'inhibiteurs de protéase au milieu de culture permet l'expression du gène *HIS3* et ainsi stimule la croissance cellulaire.

Dans le système Trp1-M (Partie II), le site de clivage de la protéase est inséré dans la chaîne polypeptidique de Trp1p, une protéine de levure essentielle à la synthèse du tryptophane. L'inactivation de cette enzyme par la protéase empêche la prolifération cellulaire en milieu sans tryptophane, alors que l'ajout d'inhibiteurs de protéase la relance. Les deux systèmes reposent ainsi sur un read-out positif et donc sélectionnent directement des composés non toxiques.

Dans la troisième partie de cette thèse, le système LexA-M a été utilisé pour un screen à large échelle visant à identifier des inhibiteurs de la protéase du cytomégalo­virus. Une librairie de 15'000 molécules a été criblée de manière entièrement automatisée. Nous avons obtenu 67 hits qui ont stimulé la prolifération de levures de

manière dose-dépendante. Ces 67 hits ont ensuite été évalués par des tests biochimiques et cellulaires. Parmi eux, 8 ont fait preuve de bonnes propriétés antivirales en culture cellulaire, mais l'interaction directe entre ces inhibiteurs putatifs et la protéase n'a pas pu être démontrée.

2. Introduction

2.1. The cytomegalovirus

Human cytomegalovirus (HCMV) is a member of the *herpesviridae* family. To date, 8 human herpes viruses are known. They are classified in 3 subfamilies: the *alphaherpesvirinae*, characterized by a variable host range, a short replicative cycle and a rapid spread in culture; the *betaherpesvirinae*, characterized by their high level of host specificity, their slow replication cycle and the spread of infection from cell to cell in cell culture; and the *gammaherpesvirinae*, characterized by a host range limited to the host family and the replication in lymphoblastoid cells (Fields, Knipe et al. 1996). Besides the cytomegalovirus, the *betaherpesvirinae* subfamily also includes human herpesvirus 6 (HHV-6) and HHV-7. *Alphaherpesvirinae* family comprises herpes simplex virus types 1 and 2 (HSV1 and HSV2), varicella-zoster virus (VZV), and HHV-8, also known as Kaposi's sarcoma related herpesvirus (KSHV). The last subfamily, the *gammaherpesvirinae*, contains the Epstein-Barr virus (EBV) (Fields, Knipe et al. 1996).

Diseases caused by these viruses are varied (Table 1), but generally benign in immunocompetent hosts: HSV1 and 2 cause respectively labial and genital herpes, VZV is responsible for chickenpox and shingles, EBV causes mononucleosis, also called *kissing disease*. In many cases, infections with these viruses are even asymptomatic. In contrast, immunocompromised patients generally suffer from much more severe affection, like herpes zoster (VZV), pneumonia (CMV), hepatitis (HSV1), and patients infected with HHV-6 and 7 often reject grafts (Wathen 2002; Coen and Schaffer 2003). Moreover, all herpesviruses severely affect embryonic development when primarily infection occurs during pregnancy (Fields, Knipe et al. 1996), causing diseases like jaundice, chorioretinitis, or permanent neurological damage (e.g. mental retardation, deafness, motor deficits).

The cytomegalovirus consists of a single molecule of linear double strand DNA with approximately 230'000 base pairs enclosed by an icosahedral capsid and an envelope (Figure 1). The mature viral particle has a diameter of 150-200 nm. A layer of amorphous material called tegument surrounds the nucleocapsid. The envelope harbors

glycoprotein complexes that are recognized by cell surface receptors (Seitz, Burger et al. 2001).

At the beginning of the virus replication cycle (Figure 2), viral glycoproteins bind to cell surface receptors, which initiates fusion of the two membranes. The capsid is internalized and transported to the nucleus. In the nucleoplasm, the genome is released and circularized. First, the early genes coding mainly for transcription factor and enzymes needed for the DNA replication are transcribed, followed by the late genes coding for capsid and teguments proteins. An empty intermediate capsid is assembled, and viral DNA is packed in it. Teguments bind to the nuclear membrane, and viral envelope is acquired by budding through it. Virions are released by transport via the endoplasmic reticulum to the cell surface (Coen and Schaffer 2003).

During the lytical phase, the virus is transmitted via cell to cell contacts to different organs, in particular salivary glands, kidney, bone marrow and peripheral blood monocytes, where it stays silent during the latency phase (Seitz, Burger et al. 2001). During that phase, the genome remains with 10 to 100 episomal copies in the nucleus of host cells, but no viral protein is produced (Fields, Knipe et al. 1996). This long-term latent infection, with periods of recurring viral replication, is a feature common to herpesviruses. Reactivation of the lytical phase is induced by different forms of stress or immune suppression.

HCMV can be transmitted via saliva, sexual contact, placental transfer, breastfeeding, blood transfusion or transplantation (Gandhi and Khanna 2004). Estimation for the seroprevalence of HCMV amounts to 50-70% in healthy adults, and up to 90% of the urban population is infected (Martinez, Castro et al. 2001). In Third World countries, the prevalence can even reach 100% (Seitz, Burger et al. 2001). But except for some cases of mononucleosis-like illness, infection with HCMV rarely causes disease in immunocompetent individuals. However, the clinical importance of this virus has increased substantially over the past 30 years due to the advent of organ transplantation and the emergence of the AIDS epidemic. Indeed, immunocompromised patients develop severe illness after the reactivation or the infection with HCMV, and they usually require antiviral treatments for a lifetime (Martinez, Castro et al. 2001).

2.2. Current antiviral treatments

Antiviral agents currently licensed for the treatment of HCMV infections are ganciclovir, valganciclovir, foscarnet, cidofovir and fomivirsen (De Clercq 2003) (See Table 1). With the exception of fomivirsen, which blocks the expression of immediate early mRNA, all these drugs are nucleoside analogues that target the viral DNA polymerase. Unfortunately for the patients affected, the clinical usefulness of these drugs is limited because they cause toxic side effects and they need to be injected either intravenously or intraocularly. Only valganciclovir is orally available, but there is no evidence that this drug is equivalent to the “gold standard” ganciclovir in terms of efficacy and safety (Preiksaitis 2005). Moreover, even if these drugs reduce viral proliferation in patients, they cannot clear the virus completely, and this predisposes to the development of viral drug resistances. HCMV strains with reduced susceptibility to ganciclovir, cidofovir and fomivirsen during long-term therapy have already been documented (Mulamba, Hu et al. 1998; Abraham, Lastere et al. 1999).

Thus, improved alternatives are needed for treating HCMV infections. While in the last decades efforts were invested mostly in nucleoside analogues that inhibit DNA polymerase, a new interest appears for drugs that target other processes necessary for the virus life-cycle: virus-induced cell fusion, viral transcription, viral protein synthesis and processing, capsid maturation, viral DNA cleavage and packaging. A number of research programs have identified novel classes of small molecules that inhibit these processes, but most of them are still in the preclinical or early clinical phases of drug development (De Clercq 2003).

2.3. The cytomegalovirus protease as a target

Herpesviruses encode a serine protease, which cleaves the assembly protein precursor (pAP), a major component of the intermediate capsid (Kan 1998). Studies on HSV-1 protease, homologous to HCMV protease, showed that this cleavage is essential for the infectivity of the virus (Preston, Coates et al. 1983; Gao, Matusick-Kumar et al. 1994). HCMV protease is expressed as a 708-amino-acid precursor, product of the open reading

frame UL80a, and cleaves autocatalytically at three consensus sequences called maturational (M), release (R) and internal (I) sites (Figure 3) (Waxman and Darke 2000). M-site cleavage of the precursor eliminates a 6-kDa carboxyl tail, which mediates interaction with the major capsid protein (MCP) at the earliest stage of capsid assembly. R-site cleavage separates the N-terminal 29 kDa proteolytic domain from the C-terminal 38 kDa scaffolding domain. In contrast to M- and R-site that are required to produce infectious virus (Gibson 1996), the I-site cleavage, which produces a two chains protease, is not essential, even though Loveland et al. recently showed that this cleavage enhances infectivity (Loveland, Chan et al. 2005). The assembly precursor (pAP) is transcribed and translated from a UL80.5 ORF nested in the UL80a, and therefore contains the same 38 kDa domain like the C-terminal scaffolding domain of pPR.

Wade Gibson (Gibson 2001) proposed a model for the function of HCMV protease in the capsid assembly (Figure 4): in the cytoplasm, the protease precursor (pPR), the assembly protein precursor (pAP) and the major capsid protein (MCP) form oligomers that translocate into the nucleus and assemble in spherical procapsid. There, the close proximity and the concentration increase of the protease precursor proteins promote their dimerization and subsequent activation. Once activated, the protease precursors cleave their own M-site and that of the assembly precursors, and cleavage products are then eliminated from the capsid cavity, making room for DNA packaging.

Whether the 29 kDa mature protease or the 75kDa protease precursor is the physiologically relevant form responsible for capsid maturation is still a matter of debate. For Robertson et al. (Robertson, McCann et al. 1996), cleavage at M- and R-sites occurs after the assembly of the procapsid in the nucleus and involves most likely the precursor form. For Waxman and Dark (Waxman and Darke 2000), the precursor form of the enzyme would rather be the initiating catalyst for subsequent processing performed by the mature protease. Most of enzymology and inhibition studies to date have been performed with the 29 kDa mature form of the protease (Pinko, Margosiak et al. 1995; Bonneau, Grand-Maitre et al. 1997; Khayat, Batra et al. 2003), though the precursor form of HCMV protease has been demonstrated to be catalytically active as well (Welch, McNally et al. 1993; Wittwer, Funckes-Shippy et al. 2002).

HCMV protease shares considerable amino acid identity with the other herpesvirus proteases: about 40% with HHV-6 (*betaherpesvirinae*), and still 26% with VZV, HSV-1 and HSV-2, (*alphaherpesvirinae*) (Qiu, Janson et al. 1997). However, herpesvirus proteases do not share any homology with classical serine proteases. X-ray crystallography studies show that they have unique backbone folds, containing a central, mostly antiparallel seven-stranded β -barrel surrounded by eight helices, and that this structural feature is well conserved among the proteases of the three herpesvirus subfamilies (Tong 2002). The active site of herpesvirus proteases is located on the surface of the β -barrel and contains a novel Ser-His-His catalytic triad. The presence of the second His residue is unprecedented, as most classical serine proteases contain an acidic residue (Asp or Glu) as the third member. This His third member makes a much smaller contribution to the catalysis by herpesvirus proteases (Khayat, Batra et al. 2001), which as a consequence are slow enzymes: HSV-1 and HCMV protease have catalytic efficiencies (k_{cat}/K_m) of $38 \text{ s}^{-1} \text{ M}^{-1}$, and $404 \text{ s}^{-1} \text{ M}^{-1}$ respectively, as compared for example with chymotrypsin, which catalytic efficiency is about $10^7 \text{ s}^{-1} \text{ M}^{-1}$ (Hall and Darke 1995).

Biochemical studies showed that herpesvirus proteases exist in solution in a monomer-dimer equilibrium, and that only the dimer is catalytically active. The HCMV protease dimer has a K_d of $8 \mu\text{M}$ in the absence of glycerol, and of 1.9 nM , in presence of 25% glycerol (Margosiak, Vanderpool et al. 1996). Indeed, it has been shown that kosmotropic agents, such as glycerol but also phosphates, sulfates or acetates, enhance protease catalytic activity by stabilizing the dimer configuration (Yamanaka, DiIanni et al. 1995; Waxman and Darke 2000). As illustrated in Figure 5, each monomer of HCMV protease has an active site, and these active sites do not interact with each other, as opposed to many other dimeric proteases where the two monomers form one active site. The HIV aspartic protease for example is a homodimer harboring its active site at the dimer interface, with each monomer contributing one of the catalytic Asp residues (Tong 2002).

Many efforts have been invested to identify cytomegalovirus protease inhibitors. Pharmaceutical companies, but also academic laboratories, worked at the development of different classes of compounds, for example benzoxazinones (Flynn, Abood et al. 1997), monobactams (Ogilvie, Yoakim et al. 1999), or naphthalene derivatives (Gopalsamy 2004).

In most cases, inhibitors target the active site by acylating the catalytic serine 132, or alternatively they oxidize the conserved cysteine 161, which is in close proximity to the active site. Recently, Borthwick et al. reported a series of transactams with IC₅₀ values in the nanomolar range in both *in vitro* and cell culture assays. These drug candidates showed no cytotoxicity and good pharmacokinetics (Borthwick, Davies et al. 2003; Borthwick 2005). However, no clinical trial was reported so far.

2.4. Biochemical versus cellular assays

Traditionally, protease activity has been measured *in vitro* with assays using the isolated protease and a purified peptide or protein substrate. All published drug discovery programs that have targeted the HCMV protease to this day have been based on *in vitro* assays. To monitor HCMV protease activity, a series of peptides have been prepared that derive from the M- or the R-cleavage site. Their lengths vary from 4 to 16 amino acids, and they are often coupled to chemical structures that allow cleavage detection by different methods: fluorescence, radioactivity or colorimetry. The amino acid sequences of these peptides substrates have been optimized to improve the k_{cat}/k_M of the reaction, leading to substrates certainly very efficiently cleaved, but also sometimes far away from the natural M- or R- cleavage sequences (Bonneau, Plouffe et al. 1998). Moreover, HCMV protease *in vitro* assay buffers contain either glycerol or kosmotropic salts (or both) in high concentrations to promote dimerization of the enzyme. Thus, *in vitro* assay conditions do not really reflect the physiological environment of the protease. This might be an explanation why most inhibitors developed so far are very potent in enzymatic assays, but poorly active in cell culture.

For the following reasons, more effort should be invested in cell-based assays to screen for HCMV protease inhibitor: 1. In the cell, protease conformation and activity are examined in a cytoplasmic context that is closer to natural physiological state than the buffers of *in vitro* assays. 2. This physiological environment also allows a selection for non-toxic compounds, which are metabolically stable and able to permeate membranes. 3. No purification of the enzyme is required, avoiding a time consuming and costly process to obtain an active target.

Ideally, compounds should be tested in human cells, since they provide the most physiologically relevant system. However, human cells are expensive to culture and difficult to propagate in automated HTS systems. Yeasts, which are prototypes of eukaryotic cells, are in comparison very convenient tools, fast growing, cheap, easy to modify genetically, and can be handled in semi-sterile conditions (Barberis 2002).

A critical limitation associated with yeast-based screening of small molecules is imposed by the lower drug sensitivity of wild-type yeast compared with higher eukaryotic cells (Rogers, Decottignies et al. 2001). This difference in drug sensitivity has been mostly attributed to very efficient compound efflux by ABC transporters that lower the intracellular concentration of compounds. However, intracellular drug accumulation in yeast can be enhanced by deleting the major ABC transporters, Pdr5p, Snq2p and Yor1 (Kolaczowski, Kolaczowska et al. 1998).

2.5. Conclusion

In this study, I describe in a first part two cellular growth selection systems for the identification of inhibitors of HCMV protease. In these systems, yeast cells expressing the HCMV protease present a slow growth phenotype, and a normal proliferation rate is restored upon inactivation of the protease activity. In a second part, I will relate how these yeast growth selection systems were used for screening a 15'000 compound library, and how the hits originally identified from that screen were tested in different biochemical and antiviral assays.

2.6 References

- Abraham, B., S. Lastere, et al. (1999). "Ganciclovir resistance and UL97 gene mutations in cytomegalovirus blood isolates from patients with AIDS treated with ganciclovir." J Clin Virol **13**(3): 141-8.
- Barberis, A. (2002). "Cell-based high-throughput screens for drug discovery." European BioPharmaceutical Review(winter).
- Bonneau, P. R., C. Grand-Maitre, et al. (1997). "Evidence of a conformational change in the human cytomegalovirus protease upon binding of peptidyl-activated carbonyl inhibitors." Biochemistry **36**(41): 12644-52.
- Bonneau, P. R., C. Plouffe, et al. (1998). "Design of fluorogenic peptide substrates for human cytomegalovirus protease based on structure-activity relationship studies." Anal Biochem **255**(1): 59-65.
- Borthwick, A. D. (2005). "Design of translactam HCMV protease inhibitors as potent antivirals." Med Res Rev **23**: 23.
- Borthwick, A. D., D. E. Davies, et al. (2003). "Design and synthesis of pyrrolidine-5,5'-trans-lactams (5-oxo-hexahydropyrrolo[3,2-b]pyrroles) as novel mechanism-based inhibitors of human cytomegalovirus protease. 4. Antiviral activity and plasma stability." J Med Chem **46**(21): 4428-49.
- Coen, D. M. and P. A. Schaffer (2003). "Antiherpesvirus drugs: a promising spectrum of new drugs and drug targets." Nat Rev Drug Discov **2**(4): 278-88.
- De Clercq, E. (2003). "New inhibitors of human cytomegalovirus (HCMV) on the horizon." J Antimicrob Chemother **51**(5): 1079-83. Epub 2003 Apr 14.
- Fields, B. N., D. M. Knipe, et al. (1996). Fields Virology. Philadelphia: Lippincott-Raven.
- Flynn, D. L., N. A. Abood, et al. (1997). "Recent advances in antiviral research: identification of inhibitors of the herpesvirus proteases." Curr Opin Chem Biol **1**(2): 190-6.
- Gandhi, M. K. and R. Khanna (2004). "Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments." Lancet Infect Dis **4**(12): 725-38.

- Gao, M., L. Matusick-Kumar, et al. (1994). "The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth." J Virol **68**(6): 3702-12.
- Gibson, W. (1996). "Structure and assembly of the virion." Intervirol **39**(5-6): 389-400.
- Gibson, W. (2001). "Action at the assemblin dimer interface. [letter; comment.]" Nat Struct Biol **8**(9): 739-741.
- Gopalsamy, A. L., K.; O'Connell J.O (2004). "Design and Syntheses of 1,6-Naphtalene Derivatives as Selective HCMV Protease Inhibitors." Journal of Medicinal Chemistry.
- Hall, D. L. and P. L. Darke (1995). "Activation of the herpes simplex virus type 1 protease." Journal of Biological Chemistry **270**(39): 22697-700.
- Kan, C.-C. (1998). Cytomegalovirus assemblin (162). Handbook of Proteolytic Enzymes. N. D. R. A.J. Barret, J.F. Waessner, academic press: 468-470.
- Khayat, R., R. Batra, et al. (2001). "Investigating the role of histidine 157 in the catalytic activity of human cytomegalovirus protease." Biochemistry **40**(21): 6344-51.
- Khayat, R., R. Batra, et al. (2003). "Structural and biochemical studies of inhibitor binding to human cytomegalovirus protease." Biochemistry **42**(4): 885-91.
- Kolaczowski, M., A. Kolaczowska, et al. (1998). "In vivo characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network." Microb Drug Resist **4**(3): 143-58.
- Loveland, A. N., C. K. Chan, et al. (2005). "Cleavage of human cytomegalovirus protease pUL80a at internal and cryptic sites is not essential but enhances infectivity." J Virol **79**(20): 12961-8.
- Margosiak, S. A., D. L. Vanderpool, et al. (1996). "Dimerization of the human cytomegalovirus protease: kinetic and biochemical characterization of the catalytic homodimer." Biochemistry **35**(16): 5300-7.
- Martinez, A., A. Castro, et al. (2001). "Recent strategies in the development of new human cytomegalovirus inhibitors." Med Res Rev **21**(3): 227-44.

- Mulamba, G. B., A. Hu, et al. (1998). "Human cytomegalovirus mutant with sequence-dependent resistance to the phosphorothioate oligonucleotide fomivirsen (ISIS 2922)." Antimicrob Agents Chemother **42**(4): 971-3.
- Ogilvie, W. W., C. Yoakim, et al. (1999). "Synthesis and antiviral activity of monobactams inhibiting the human cytomegalovirus protease." Bioorg Med Chem **7**(8): 1521-31.
- Pinko, C., S. A. Margosiak, et al. (1995). "Single-chain recombinant human cytomegalovirus protease. Activity against its natural protein substrate and fluorogenic peptide substrates." Journal of Biological Chemistry **270**(40): 23634-40.
- Preiksaitis, J. K. (2005). "Valganciclovir treatment of cytomegalovirus disease: a promising but not yet fully validated therapeutic approach." Am J Transplant **5**(7): 1783.
- Preston, V. G., J. A. Coates, et al. (1983). "Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA." J Virol **45**(3): 1056-64.
- Qiu, X., C. A. Janson, et al. (1997). "Crystal structure of varicella-zoster virus protease." Proceedings of the National Academy of Sciences of the United States of America **94**(7): 2874-9.
- Robertson, B. J., P. J. McCann, 3rd, et al. (1996). "Separate functional domains of the herpes simplex virus type 1 protease: evidence for cleavage inside capsids." Journal of Virology **70**(7): 4317-28.
- Rogers, B., A. Decottignies, et al. (2001). "The pleiotropic drug ABC transporters from *Saccharomyces cerevisiae*." J Mol Microbiol Biotechnol **3**(2): 207-14.
- Seitz, R., R. Burger, et al. (2001). "Human Cytomegalovirus." Infus Ther Transfus med(28): 165-171.
- Tong, L. (2002). "Viral proteases." Chem Rev **102**(12): 4609-26.
- Wathen, M. W. (2002). "Non-nucleoside inhibitors of herpesviruses." Rev Med Virol **12**(3): 167-78.
- Waxman, L. and P. L. Darke (2000). "The herpesvirus proteases as targets for antiviral chemotherapy." Antiviral Chemistry & Chemotherapy **11**(1): 1-22.

- Welch, A. R., L. M. McNally, et al. (1993). "Herpesvirus proteinase: site-directed mutagenesis used to study maturational, release, and inactivation cleavage sites of precursor and to identify a possible catalytic site serine and histidine." J Virol **67**(12): 7360-72.
- Wittwer, A. J., C. L. Funckes-Shippy, et al. (2002). "Recombinant full-length human cytomegalovirus protease has lower activity than recombinant processed protease domain in purified enzyme and cell-based assays." Antiviral Research **55**(2): 291-306.
- Yamanaka, G., C. L. DiIanni, et al. (1995). "Stimulation of the herpes simplex virus type I protease by antichaeotropic salts." Journal of Biological Chemistry **270**(50): 30168-72.

2.7. Figures

Table 1. Herpesvirus diseases and treatments

Virus	Associated diseases		Marketed antivirals
	Normal host	Immunocompromised host	
HSV-1	Herpes labialis (cold sores)	Disseminated herpes	Acyclovir Penciclovir
HSV-2	Genital herpes	Disseminated herpes	Acyclovir Valaciclovir Famciclovir
VZV	Chicken pox Herpes zoster	Herpes zoster	Acyclovir Valaciclovir Famciclovir
CMV	Congenital CMV disease	Retinitis Pneumonia GI disease Graft rejection	Ganciclovir Valganciclovir Foscarnet Cidofovir Formivirsen
EBV	Infectious mononucleosis	Lymphomas (PTLD)	None
HHV-6	Exanthem subitum	Graft rejection	None
HHV-7	Exanthem subitum	Graft rejection	None
HHV-8	Kaposi's sarcoma	Kaposi's sarcoma	None

From Wathen, M. W. (2002). "Non-nucleoside inhibitors of herpesviruses." *Rev Med Virol* 12(3): 167-78.

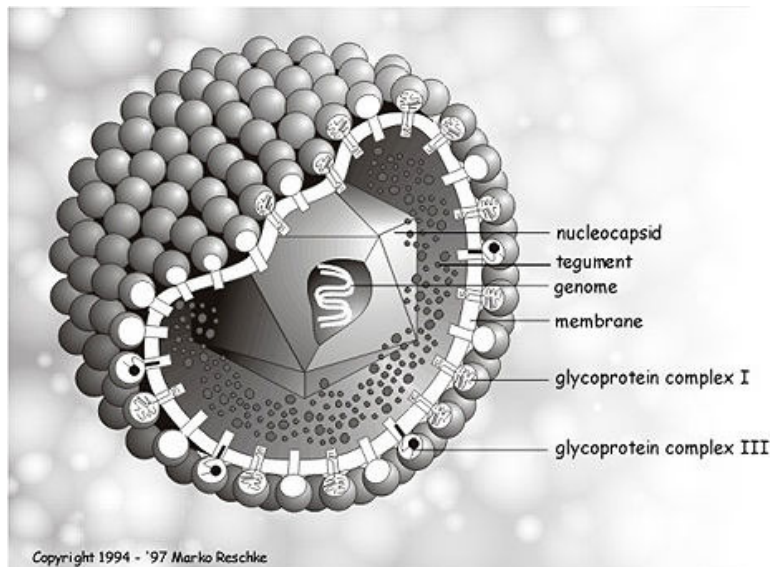


Figure 1: Schematic drawing of human cytomegalovirus

From Gandhi, M. K. and R. Khanna (2004). "Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments." *Lancet Infect Dis* 4(12): 725-38.

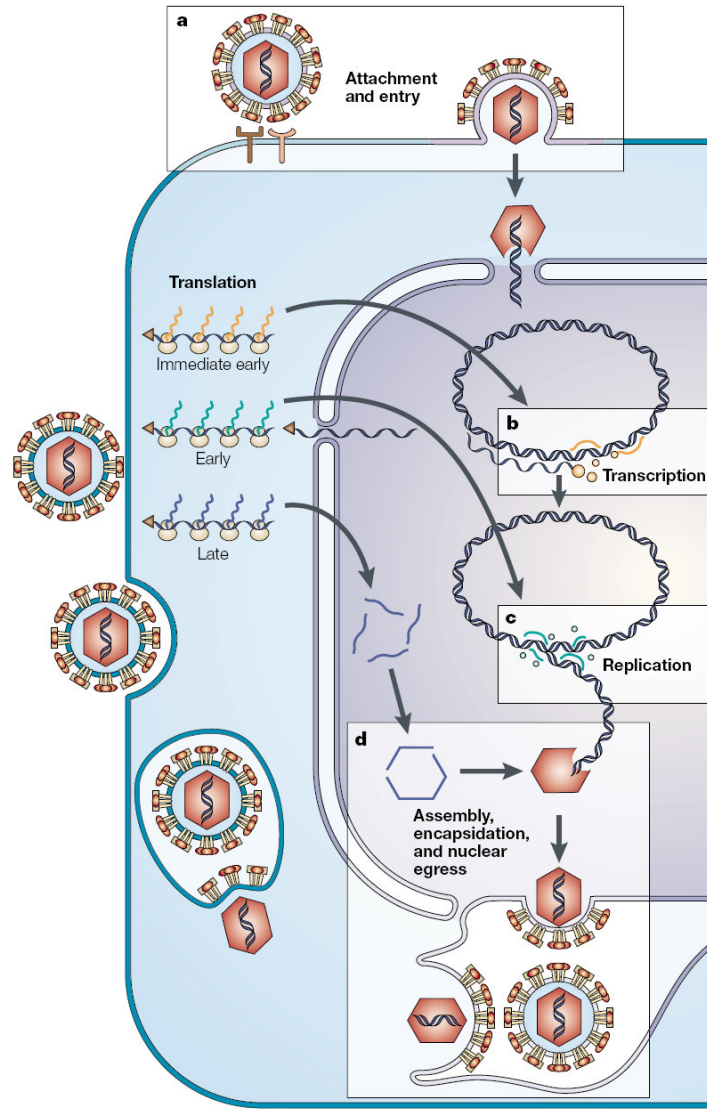


Figure 2. Herpesvirus replication cycle.

a. Attachment and entry. Viral membrane proteins on virus particles bind to cellular receptors on the plasma membrane of the cell, which initiates fusion of the two membranes. Nucleocapsids containing the viral genome (red hexagons) are liberated into the cytoplasm and transported to nuclear pores. Viral DNA is released into the nucleus and circularizes. **b.** Transcription. Three classes of viral genes are transcribed and translated into proteins. Immediate-early proteins (yellow) participate in further transcription. **c.** Replication. Early proteins (green) synthesize new viral DNA molecules using circularized input DNA as a template. **d.** Assembly, encapsidation and nuclear egress. Late proteins (blue) assemble into capsids, which incorporate newly replicated viral DNA. Nucleocapsids leave the nucleus by budding through the inner nuclear membrane (a process termed 'envelopment') into the perinuclear space. Through a complex process of de- and re-envelopment, mature virus particles reach exocytic vesicles, which fuse with the plasma membrane and release new virus particles into the extracellular space.

From Coen, D. M. and P. A. Schaffer (2003). "Antiherpesvirus drugs: a promising spectrum of new drugs and drug targets." *Nat Rev Drug Discov* 2(4): 278-88.

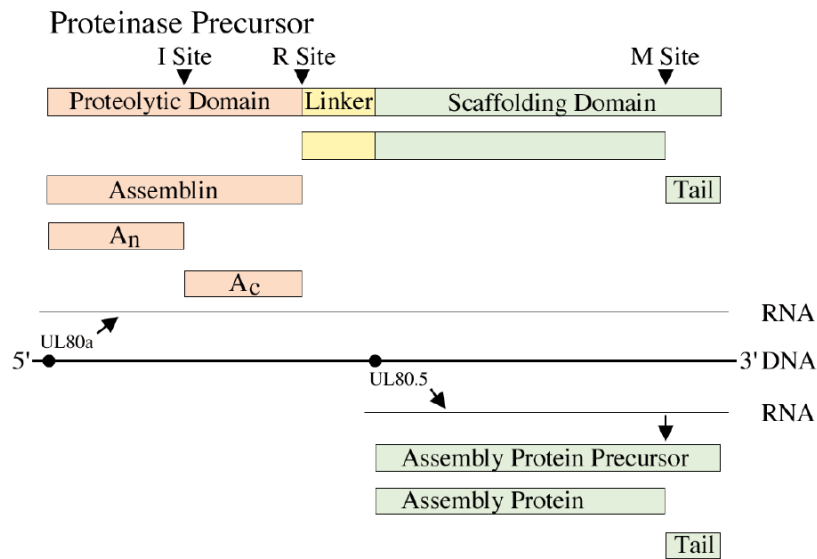


Figure 3. CMV assemblin is derived from a precursor.

The proteinase precursor is encoded by open reading frame UL80a and is autoproteolytically cleaved at the maturational (M), release (R), and internal (I) sites. M site cleavage removes the carboxyl 'Tail' and R site cleavage releases the proteolytic domain, assemblin. I-site cleavage converts assemblin monomers to dimers composed of its amino (An) and carboxyl (Ac) halves. UL80.5 encodes the protease substrate, the assembly protein precursor, and is nested, in frame, and 3'-coterminal with UL80a. The amino acid sequence of the assembly protein precursor is identical to that of the proteinase scaffolding domain.

From Gibson, W. (2001). "Action at the assemblin dimer interface." *Nat Struct Biol* **8**(9): 739-741.

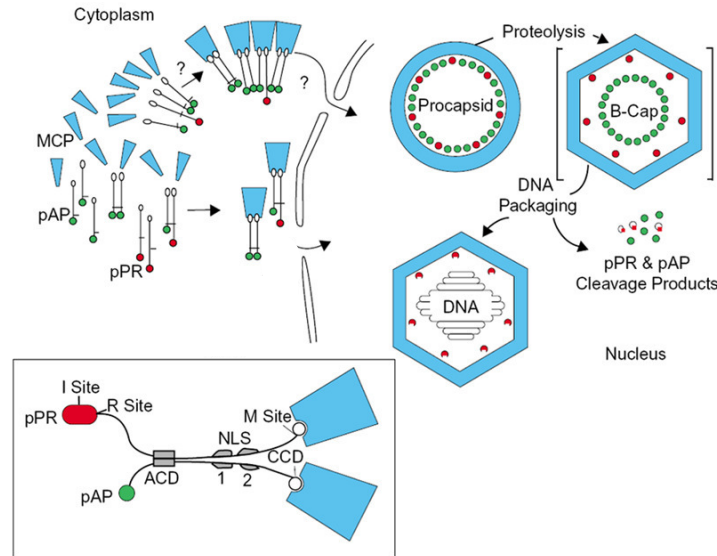


Figure 4. CMV protease role during capsid maturation.

Depicted here are cytoplasmic interactions between the proteinase precursor (pPR, red), assembly protein precursor (pAP, green), and major capsid protein (MCP, blue) to form oligomers that are competent for nuclear translocation and procapsid assembly. The procapsid scaffolding structure (beaded, green/red) underlying the shell (blue ring) undergoes rearrangement following proteolytic cleavage and capsid angularization. Only a portion of the proteinase precursor remains in the mature, DNA-containing capsid. Inset shows functional sequences shared by pPR and pAP: amino conserved domain (ACD), nuclear localization signals (NLS) 1 and 2, carboxyl conserved domain (CCD), and maturational (M) site.

From Gibson, W. (2001). "Action at the assemblin dimer interface." *Nat Struct Biol* **8**(9): 739-741.

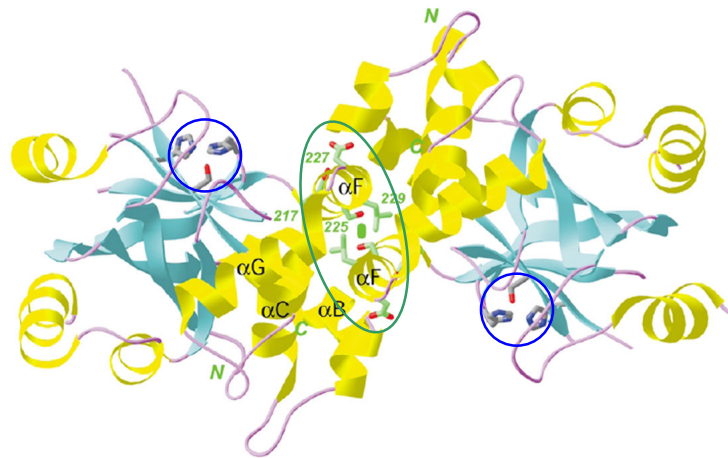


Figure 5. Schematic drawing of the dimer of HCMV protease.

The Ser-His-His catalytic triads are highlighted in blue circles, and the dimer interface in the green oval.

From Batra, R., R. Khayat et al. (2001). "Molecular mechanism for dimerization to regulate the catalytic activity of human cytomegalovirus protease." *Nat Struct Biol* **8**(9)(Sept.): 810-7.

3.1. Part I

A yeast growth selection system based on reporter gene activation to detect human cytomegalovirus protease activity

LexA-M system

Abstract

The proteolytic processing of the human cytomegalovirus (HCMV) assembly protein is an essential step in virion maturation. Here we describe a genetic system in the yeast *Saccharomyces cerevisiae* that monitors the activity of the protease responsible for this cleavage. We take advantage of the properties of the yeast transcriptional activator Gal4p, which contains two separable and functionally essential domains: the amino-terminal binding domain and the carboxy-terminal transcriptional activating domain. In our system, the cleavage M-site is inserted between these two domains of Gal4p. Thus, proteolysis by HCMV protease inactivates the transcription factor that controls the expression of the *HIS3* reporter gene, and thereby suppresses yeast survival in minimal medium lacking histidine. Cell proliferation in selective medium is only possible when the protease is inhibited. This Part I presents key steps in the development of a system for the identification of HCMV protease inhibitors.

1. Material and Methods

1.1. Yeast strains

The YDE172 strain, which carries the divergently oriented *LacZ* and *HIS3* genes under the control of the natural UAS_G from the *GAL1-GAL10* regulatory sequences integrated in the *his3Δ200* locus, was already described elsewhere (Auf der Maur, Escher et al. 2001).

The VCY3 strain is derived from the RLY07 strain (*MATa ura3-52 his3Δ200 leu2Δ1 trp1Δ63 lys2Δ385 Δpdr5 Δsnq2 Δyor1*). To obtain VCY3 the integrating plasmid pAdMO2R, which carries the divergently oriented *LacZ* and *HIS3* genes under the control of a bi-directional promoter with 6 LexA-binding sites, was linearized at the *Afl*III site in the *HIS3* 3' untranslated region and integrated into the *his3Δ200* locus of RLY07. To allow the selection of transformants on –His plates, the plasmid pMix02, encoding a LexA-Gal4AD transactivator construct and a Ura3p marker, was previously transformed in RLY07, and subsequently eliminated by selection on plates containing 0.1% 5-fluoroorotic acid (5-FOA). Correct integration of the cassette and loss of pMix02 plasmid were confirmed by genomic PCR and functional assays.

Yeast transformation was performed according to the standard lithium acetate protocol (Gietz, St Jean et al. 1992).

1.2. Construction of plasmids

The pAdMO2R plasmid was obtained by replacing the natural *HIS3* promoter in the pDE200 integrating plasmid (Escher, Bodmer-Glavas et al. 2000) with the tight *LEU2* promoter, via *Sal*I and *Spe*I restriction enzymes. The pMix02 plasmid was obtained by subcloning the LexA (1-202) -Gal4AD (768-881) transactivator in a 2 micron (2μ) plasmid, under the control of an *ACT4* promoter.

The HCMV protease cleavage sequence GGVVNA↓SCRLAGG, derived from the M-site of the 75 kDa protease precursor, was flanked with unique *Nco*I and *Not*I sites, and inserted between the Gal4 (1-147) binding domain and the Gal4 (768-881) activating domain by PCR, resulting in the Gal4BD-M-Gal4AD construct. This construct was cloned via *Xba*I and *Sal*I in the pMH28 CEN4-ARS1 plasmid, which contains a

truncated *ADHI* promoter, a *GALII* terminator and a *TRPI* marker. For the experiments in the VCY3 strain, the sequence coding for Gal4BD (1-147) has been replaced via *XbaI* and *NcoI* restriction enzymes by the LexA (1-202) coding region, resulting in the LexA-M-Gal4AD construct. In order to generate the LexA-Me-Gal4AD construct (Me for M extended), the 13 amino acid cleavage sequence was replaced via *NcoI* and *NotI* sites by an extended M-type sequence consisting of 39 amino acids. The LexA-Me*-Gal4AD construct bears the mutation A→E at the position P1 of the cleavage site. The *UL80a* gene, coding for the 708 amino acids protease precursor, was obtained by PCR from HCMV infected MRC5 human cells and subcloned via unique *XbaI* and *NotI* sites in CEN4-ARS1 or 2 μ plasmids carrying a *URA3* marker and an *ACT4* promoter. The HCMV protease gene encoding amino acids 1-256 of the 708 amino acids protease precursor was obtained by PCR, using the *UL80a* gene as template, and subcloned via unique *XbaI* and *NotI* sites in the same plasmids.

1.3. Yeast media and transformation

Yeast genetic techniques and media were as described (Burke, Dawson et al. 2000). Transformation of yeast cells was performed following the lithium acetate method (Gietz, St Jean et al. 1992).

1.4. β -galactosidase assay

The β -galactosidase assay in solution was performed using permeabilized cells as described (Burke, Dawson et al. 2000). Activity was normalized to the number of cells assayed, which was measured by light scattering (OD₆₀₀). Each experiment was performed twice.

1.5. Liquid growth assay

YDE172 or VCY3 cells transformed with the plasmids indicated for each experiment in the result part were inoculated in 3 mL of 2% glucose -ura -trp drop-out medium and grown at 30°C to OD₆₀₀ 1. They were then washed with 5 mL H₂O, resuspended in the 2% glucose -ura -trp -his assay medium supplemented with 10 mM or 30 mM 3-AT and/or 10% glycerol, and diluted to a defined start OD₆₀₀ (exact medium composition and

start OD₆₀₀ are described for each experiment in the result part). At time zero, assay cultures were distributed in 96-well microtiter plates (Greiner, # 655101), with a volume of 150 µL per well, and incubated without shaking at 30°C. At the time points indicated in the results section, plates were shaken 30 s at 1300 rpm to resuspend cells and submitted to light scattering measurement at 595 nm in a Tecan Genios reader for determining cell density. All assays were conducted with triplicate or quadruplet samples and were repeated at least once.

2. Results

2.1. The principle of the HCMV protease Gal4-M growth selection system

The system that we describe here derives from a reporter system previously published that monitors HCMV protease activity (Lawler and Snyder 1999). To establish this system, authors exploited in mammalian cells the two-domain structural property of the yeast Gal4p transcription factor in such a way that the cleavage M-site was inserted between the two domains of the yeast Gal4p transcription factor. Proteolysis of the hybrid substrate interrupted the production of the luciferase reporter protein.

In our yeast Gal4-M system like in Lawler and Snyder's system, M-site is inserted between the two domains of Gal4p (Figure 1A). Protease activity separates the DNA-binding domain from the activating domain, causing a transcriptional stop of the yeast *HIS3* growth marker (Figure 1B). Thus, cells expressing the Gal4BD-M-Gal4AD construct alone are expected to proliferate normally in medium lacking histidine, whereas cells co-expressing the HCMV protease should stop growing due to the inactivation of the Gal4 transcription factor by the protease, which results in the lack of expression of the *HIS3* reporter gene.

2.2. HCMV protease domain (assemblin), but not the 75 kDa protease precursor, is active in the YDE172 strain

In order to reproduce in yeast the system established by Lawler and Snyder in mammalian cells (Lawler and Snyder 1999), we expressed the 75 kDa protease precursor (pPR, encoded by the *UL80a* gene) and the 13 amino acid cleavage sequence fused between the two Gal4p domains in the YDE172 strain. The YDE172 strain contains a bi-directional promoter that drives the expression of two reporter genes: *HIS3* and *LacZ*. To evaluate the potential of i) the Gal4BD-M-Gal4AD construct to activate the reporter gene and ii) the 75 kDa pPR to cleave the substrate sequence, we measured reporter gene expression by performing a β -galactosidase assay. Figure 2A shows that the Gal4-M substrate clearly activated the *LacZ* reporter. However, no reduction of signal was observed upon co-expression of the pPR, meaning either that this enzyme was not functional, or that it could not cleave its substrate for sterical reasons. In another

approach to detect a cleavage of the Gal4-M substrate, we expressed in YDE172 the assemblin instead of the pPR. This 29 kDa mature form of the protease was able to cleave the Gal4-M substrate and, furthermore, the cleavage rate was proportional to the concentration of protease present in the cell. Indeed, as shown on the Figure 2A, the β -galactosidase activity induced by the Gal4-M construct was diminished by 46% upon addition of the protease expressed from an *ACT4* CEN4-ARS1 (see appendix) plasmid, and by 72% when the protease was expressed from an *ACT4* 2 μ (see appendix) plasmid.

However, β -galactosidase assays are not feasible in a fully automated format, whereas growth assays are. Therefore, the transformed cells described above for the β -galactosidase assay were diluted to a start OD₆₀₀ 0.03 and incubated in medium lacking histidine during 24 hours. Cells expressing the protease precursor were not tested considering that this form of the protease displayed no activity in the β -galactosidase assay. As opposed to the results shown in Figure 2A, the assemblin in the growth assay showed only little activity. When the protease was expressed from an *ACT4* CEN4-ARS1 plasmid, no growth reduction was observed, and only 30% growth reduction could be measured when the protease was expressed from an *ACT4* 2 μ plasmid (Figure 2B).

2.3. A new VCY3 strain with a tight HIS promoter allows a better detection of HCMV protease activity in growth assay

In order to improve the detection of HCMV protease activity, a new yeast strain, VCY3, was engineered. In this new strain, the natural *HIS3* promoter, which exhibits basal expression, was replaced by the tight *LEU2* promoter, what greatly improved the signal-to-noise ratio. Moreover, 6 LexA binding sites replaced the Gal4 UAS of the YDE172. Consequently, we replaced the Gal4 binding domain by the LexA protein in the substrate construct, thus generating the new LexA-M-Gal4AD construct.

In the VCY3 strain, the detection in liquid growth assay of LexA-M-Gal4AD substrate cleavage by HCMV protease was improved. Upon co-expression of the substrate with the protease expressed from an *ACT4* CEN4-ARS1 plasmid (Figure 3), a reduction of about 20% of cell proliferation was observable after 24 hours incubation in selective medium, whereas at the same expression level no protease activity was detected in YDE172. And when the protease was expressed from a 2 μ plasmid, growth reduction

reached 62%, in opposition to 30% in the YDE172 strain. Thus, the new VCY3 strain provides a more sensitive read-out for the *HIS3* reporter gene than YDE172. In the last column of Figure 3, a control experiment is depicted where an enzymatically inactive version of HCMV protease carrying the S132A mutation at the active site serine (Chen, Tsuge et al. 1996) is investigated. As expected, no growth reduction was observed, meaning that this mutant was not able to cleave the LexA-M-Gal4AD substrate. This experiment indicates that the growth inhibition observed in presence of active protease was indeed due to the catalytic activity of the protease, and not to any toxic effect of the protein.

2.4 . Addition of glycerol increases protease activity

In the previous experiment, cell proliferation was significantly reduced upon expression of the protease from a 2 μ plasmid. However, 2 μ plasmids are not suitable for high-throughput screening, since they are present in variable quantities inside the cells after the transformation, and thus produce diverse protease amounts in each cell. In contrast, CEN4-ARS1 plasmids are present in single exemplar in each transformed cell and guaranty constant levels of protease expression.

In order to promote protease activity and thus get a sufficient reduction of cell proliferation upon protease expression from a CEN4-ARS1 plasmid, glycerol was added to yeast cultures. This substance had been shown to improve protease activity *in vitro* by promoting dimerization of the protease (Margosiak, Vanderpool et al. 1996). While in the absence of glycerol the protease expression from an *ACT4* CEN4-ARS1 plasmid resulted in a growth reduction of about 20%, with 10% glycerol added to the medium cell proliferation was reduced by 54% (Figure 4). Upon expression of the protease from a 2 μ plasmid, growth reduction in the presence of 10% glycerol was about 95%, compared to 62% reduction in the absence of glycerol. Cells transformed with the LexA-M-Gal4AD transactivator alone and grown in selective medium containing 10% glycerol exhibited 20% growth reduction in comparison to the same cells grown medium containing no glycerol. This mild toxic effect of glycerol is not detrimental to the assay.

2.5. A longer cleavage site facilitates cleavage by the HCMV protease

In a further attempt to improve HCMV protease cleavage efficiency, we replaced the 13 amino acid cleavage site by a longer 39 amino acid sequence derived from the M-site of the protease precursor protein. This exchange had a double effect: on one side, growth induced by the LexA-Me-Gal4AD (Me stands for M elongated) transactivator was increased about 3 folds, and on the other side cleavage efficiency was more than doubled. As illustrated in Figure 5, the transactivator construct bearing the 39 amino acid cleavage site induces a faster cell proliferation than the construct containing only 13 amino acids, maybe because a longer linker between LexA and Gal4AD allows a more optimal folding of the hybrid transcription factor. However, when the *ACT4* CEN4-ARS1 plasmid encoding the HCMV protease is co-expressed with these two constructs, cell proliferation is comparable. Thus, whereas the cleavage of the transactivator bearing the short M-site reduced growth of 54%, the cleavage of the construct with the long M-site led to a growth reduction of 80%. Two hypothesis could explain the importance of using a longer cleavage site: i) more than P5-P5' residues participate in the substrate recognition ii) the longer insert provides a favorable conformation to LexA-Me-Gal4AD substrate, where M-site is more accessible to HCMV protease catalytic site. To show that the cleavage occurs specifically at the M site and not in the LexA or Gal4AD domains, we substituted the alanine of the scissile bond with glutamic acid, a mutation known to prevent cleavage (Welch, McNally et al. 1993). As expected, this mutated substrate was not cleaved by the protease.

In conclusion, several optimization steps were necessary to obtain a satisfying read-out to monitor HCMV protease activity. The choice of the protease domain, of the reporter strain, of the medium composition and of the cleavage site sequence were crucial to create a sufficient ratio between the density of cells expressing substrate alone and the density of cells co-expressing the protease. Figure 6 illustrates the growth curves of these two types of cells inoculated in selective medium containing 10% glycerol and 10 mM 3-AT, at a start OD₆₀₀ of 0.01.

3. Discussion

This Part I of my PhD thesis describes the elaboration of a growth selection system that monitors human cytomegalovirus protease activity. This yeast system, inspired of previously described cell-based assays (Dasmahapatra, DiDomenico et al. 1992; Lawler and Snyder 1999), is based on the protease-mediated inactivation of a transcription factor that drives the expression of the *HIS3* reporter gene. Cells expressing the protease exhibit a slow growth phenotype, and the inhibition of protease activity by small molecules is expected to stimulate cell proliferation. Thus, the assay developed here has a positive read-out, which should avoid the selection of toxic compounds because they inhibit cell proliferation *per se*. This should reduce the number of false positives that would show up in an assay with a negative readout.

As described in the Result section, the optimization of this yeast assay monitoring HCMV protease was a long process. The protease activity was difficult to display prominently, mainly because of its low catalytic efficiency and of its characteristic of obligate dimer with high dissociation constant ($8 \mu\text{M } K_d$) (Margosiak, Vanderpool et al. 1996). Thus, we assume that proteases with higher catalytic efficiency would be easier to address in a similar yeast assay. This is indeed the case, at least for the coxsackievirus (CV) protease 3C. In parallel to the development of the reporter system for the HCMV protease we elaborated a similar system for the CV protease 3C, which is an enzyme affiliated to the chymotrypsin-like proteases (Sarkany and Polgar 2003). In our first attempt of expressing protease 3C, protease activity produced a 10 folds reduction of cell proliferation (data not shown). No artifice was necessary to improve either the protease catalytic efficiency or the assay read-out.

Once that all parameters of the HCMV protease screening assay were established we could easily adapt the system for other herpesviral proteases. As already mentioned in the introduction, herpesviral proteases are highly homologous and rely on the same mechanism of action (Waxman and Darke 2000). We expressed in our system the herpes simplex virus 1 (HSV-1) protease and the varicella-zoster virus (VZV) protease, modifying no parameter except of course that we inserted the respective M-sites between LexA and Gal4AD. Interestingly, HSV-1 protease, which has a low catalytic efficiency ($38 \text{ s}^{-1} \text{ M}^{-1}$ vs $404 \text{ s}^{-1} \text{ M}^{-1}$ for HCMV protease) (Waxman and Darke 2000), was also less

efficient in cleaving the LexA-M_{HSVp}-Gal4AD substrate than the HCMV protease to cleave its own substrate. In contrast, at a similar expression level VZV protease was much more active than HCMV protease (data not shown). I could not find any indication of catalytic efficiency for VZV protease in the literature, but I would deduce from our experiments that its k_{cat}/K_m is superior to $404 \text{ s}^{-1} \text{ M}^{-1}$.

The yeast reporter gene assay described in this Part I is configured to detect downstream transcriptional consequences of HCMV protease activity and inhibition. The read-out - cell proliferation - is simple, and the use of a reporter system provides an amplified signal. However, like all reporter gene assays, also this one is subject to non-specific interference by compounds that do not act directly on the protease but target intermediary signaling proteins of the *HIS3* pathway. Such compounds could for example prevent the LexA dimerization or its binding to the regulatory promoter region, inhibit *HIS3* transcription or translation, block His3p enzyme. In order to reduce the number of false positives, I developed an alternative assay, in which the reporter protein inactivated by the protease would be directly acting on the read-out system, thus skipping the steps of gene expression. This system is described in the second part of this thesis.

4. References

- Auf der Maur, A., D. Escher, et al. (2001). "Antigen-independent selection of stable intracellular single-chain antibodies." FEBS Lett **508**(3): 407-12.
- Burke, D., D. Dawson, et al. (2000). Methods in Yeast Genetics. Cole Spring Harbor, NY.
- Chen, P., H. Tsuge, et al. (1996). "Structure of the human cytomegalovirus protease catalytic domain reveals a novel serine protease fold and catalytic triad." Cell **86**(5): 835-43.
- Dasmahapatra, B., B. DiDomenico, et al. (1992). "A genetic system for studying the activity of a proteolytic enzyme." Proc Natl Acad Sci U S A **89**(9): 4159-62.
- Escher, D., M. Bodmer-Glavas, et al. (2000). "Conservation of glutamine-rich transactivation function between yeast and humans." Mol Cell Biol **20**(8): 2774-82.
- Gietz, D., A. St Jean, et al. (1992). "Improved method for high efficiency transformation of intact yeast cells." Nucleic Acids Res **20**(6): 1425.
- Lawler, J. F., Jr. and S. H. Snyder (1999). "Viral protease assay based on GAL4 inactivation is applicable to high- throughput screening in mammalian cells." Anal Biochem **269**(1): 133-8.
- Margosiak, S. A., D. L. Vanderpool, et al. (1996). "Dimerization of the human cytomegalovirus protease: kinetic and biochemical characterization of the catalytic homodimer." Biochemistry **35**(16): 5300-7.
- Sarkany, Z. and L. Polgar (2003). "The unusual catalytic triad of poliovirus protease 3C." Biochemistry **42**(2): 516-22.
- Waxman, L. and P. L. Darke (2000). "The herpesvirus proteases as targets for antiviral chemotherapy." Antiviral Chemistry & Chemotherapy **11**(1): 1-22.
- Welch, A. R., L. M. McNally, et al. (1993). "Herpesvirus proteinase: site-directed mutagenesis used to study maturational, release, and inactivation cleavage sites of precursor and to identify a possible catalytic site serine and histidine." J Virol **67**(12): 7360-72.

5. Figures

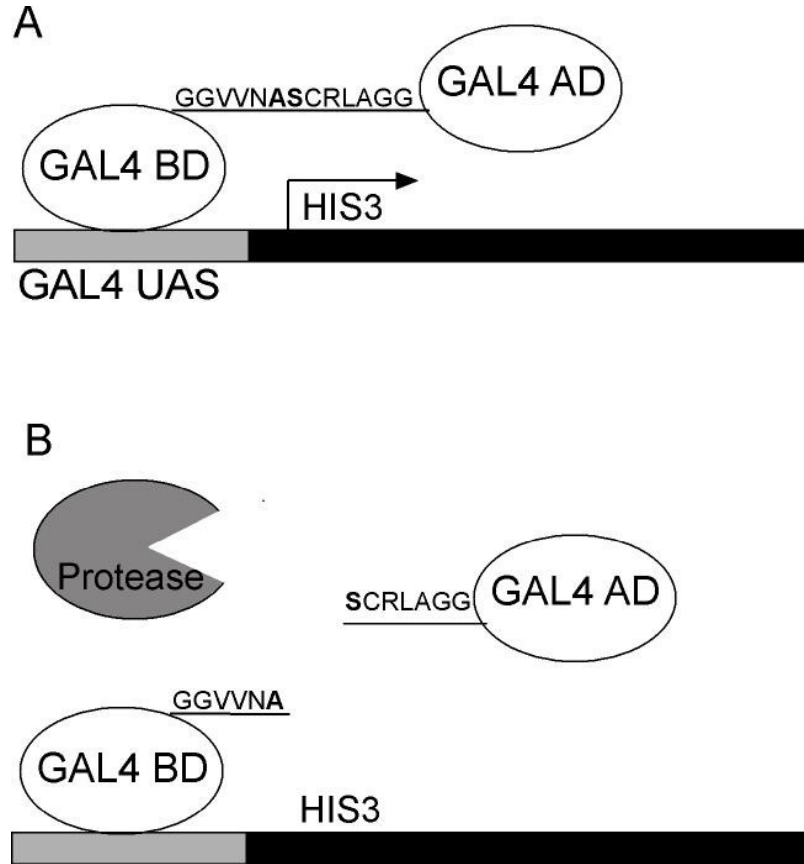


Figure 1. Schematic model of the assay.

A. The intact transcription factor binds to the *GAL4 UAS* and directs *HIS3* reporter transcription. B. In the presence of an active protease, the transcription factor is cleaved and functionally inactive. GAL4 BD: GAL4 binding domain (1-147); GAL4 AD: GAL4 activating domain (763-881); GAL4 UAS: GAL4 upstream activating sequence; GGVTNASCRLAGG: CMV protease consensus cleavage sequence.

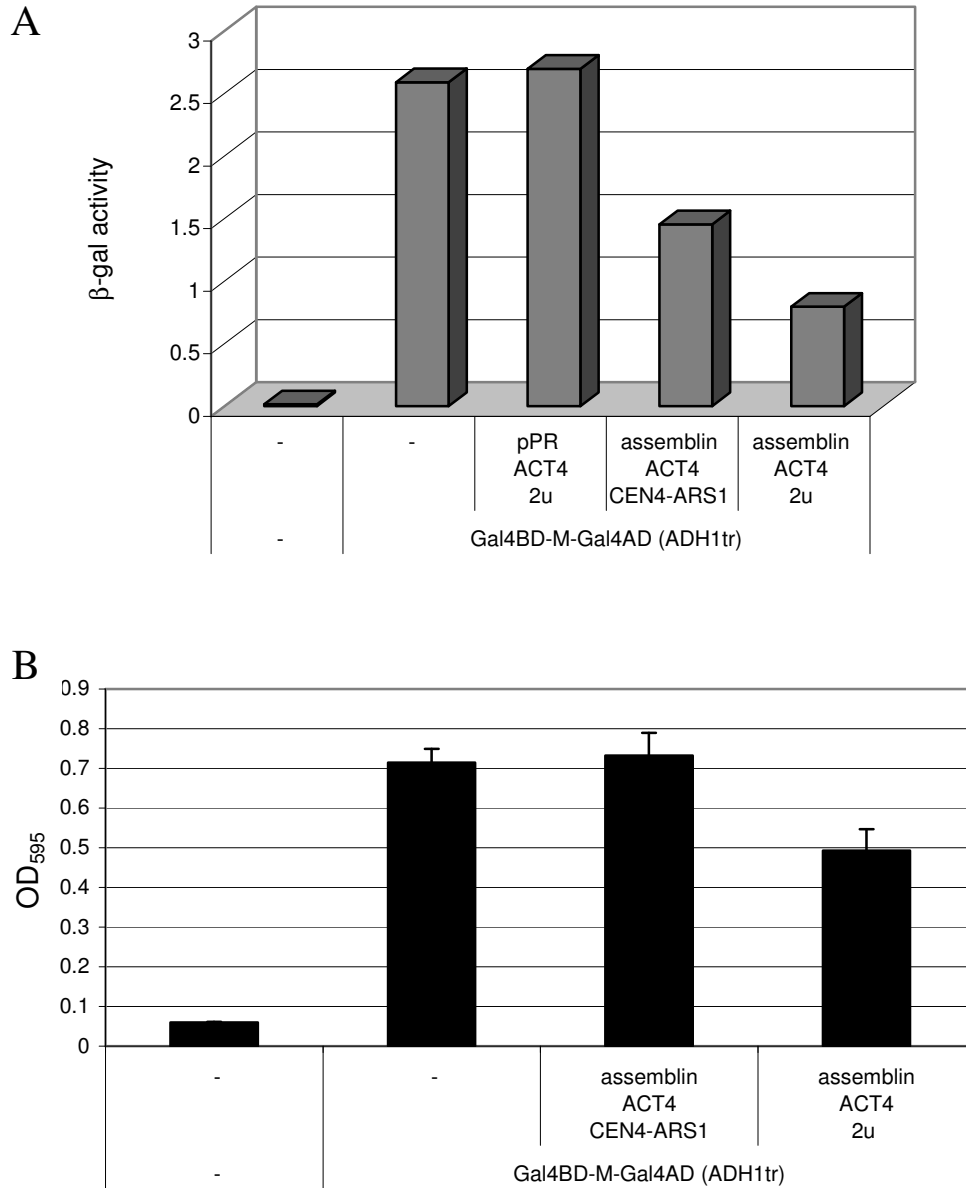


Figure 2. Assemblin activity, but not pPR activity is detected in YDE172 strain, and β -galactosidase assay is more sensitive than growth assay.

Plasmids expressing the Gal4BD-M-Gal4AD substrate or an empty plasmid and the 29 kDa mature HCMV protease (=assemblin), the 75 kDa protease precursor or an empty plasmid were co-transformed in YDE172 strain, as indicated. A. A liquid β -galactosidase assay was performed. B. Cells were diluted to a start OD₆₀₀ 0.03 and grown in selective – His –Ura –Trp medium containing 30 mM 3-AT and OD₅₉₅ was measured after 24 h.

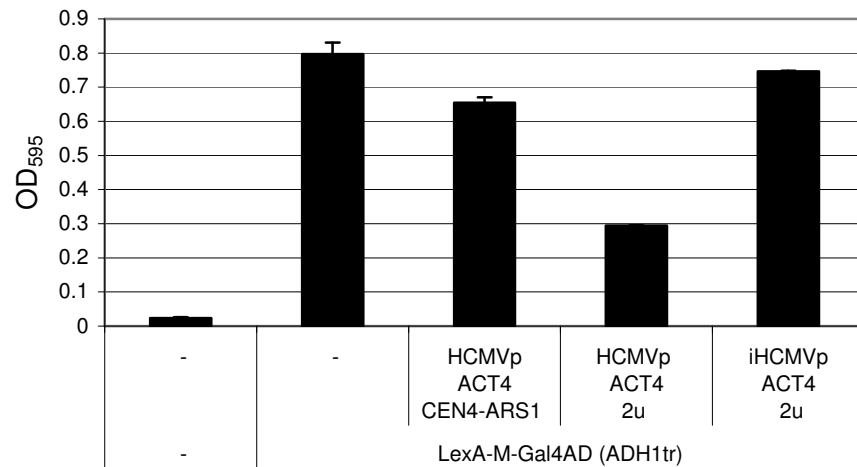


Figure 3. HCMV protease activity is better detected in VCY3 strain.

VCY3 strain was transformed with the LexA-M-Gal4AD substrate or an empty plasmid and the active or inactive HCMV protease, or an empty plasmid, as indicated. Cells were diluted to a start OD₆₀₀ 0.01 and grown in selective –His –Ura –Trp medium containing 10 mM 3-AT and OD₅₉₅ was measured after 24 h.

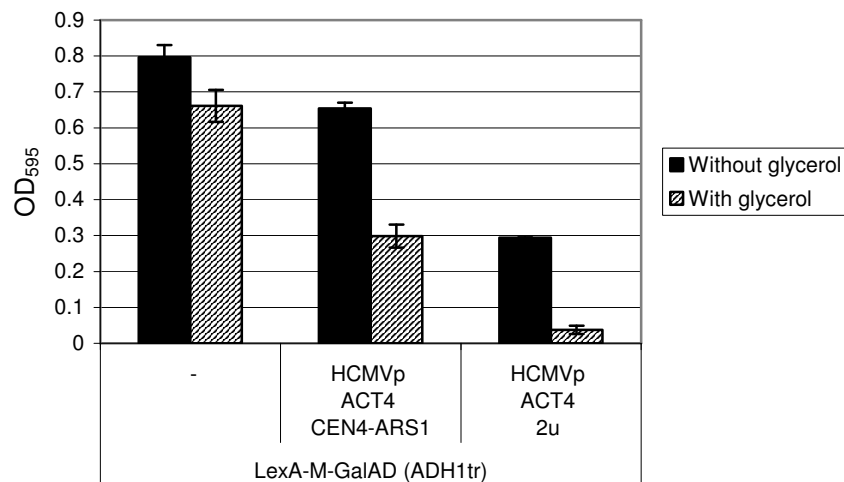


Figure 4. 10% glycerol promotes HCMV protease activity in VCY3 strain.

LexA-M-Gal4AD substrate and the HCMV protease or an empty plasmid were transformed in VCY3, as indicated. Cells were diluted to a start OD₆₀₀ 0.01 and grown in selective –His –Ura –Trp medium containing 30 mM 3-AT, in presence or in absence of 10% glycerol. OD₅₉₅ was measured after 36 h.

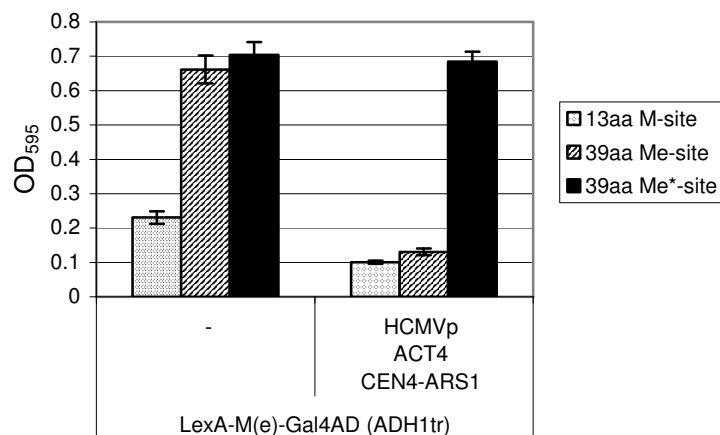


Figure 5. Cleavage is site-specific and more efficient with a long recognition sequence. Plasmids expressing LexA-M-Gal4AD, LexA-Me-Gal4AD and LexA-Me*-Gal4AD substrates were co-transformed with plasmid expressing HCMV protease or with an empty plasmid in VCY3, as indicated. Cells were diluted to a start OD₆₀₀ 0.01 and grown in selective –His –Ura –Trp medium containing 10 mM 3-AT and 10% glycerol. OD₅₉₅ was measured after 32 h.

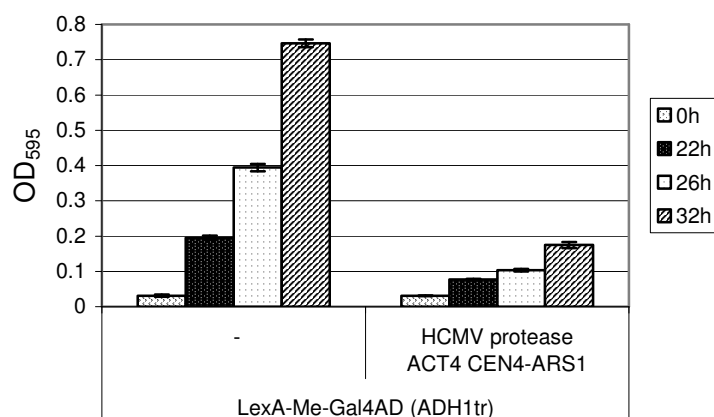


Figure 6. At the final conditions described below, cells expressing HCMV protease proliferate 4 times slower than cells expressing the LexA-Me-Gal4AD substrate alone. Plasmid expressing the LexA-Me-Gal4AD was co-transformed with plasmid expressing the HCMV protease or with an empty plasmid in VCY3, as indicated. Cells were diluted to a start OD₆₀₀ 0.01 and grown in selective –His –Ura –Trp medium containing 10 mM 3-AT, 10% glycerol and 1% DMSO. OD₅₉₅ was measured at the indicated time points.

3.2 Part II

Novel Yeast Cell-based Assay to Screen for Inhibitors of Human Cytomegalovirus Protease in a High-Throughput Format

Trp1-M system

Abstract

The protease encoded by the human cytomegalovirus (HCMV) is an attractive target for antiviral drug development because of its essential function in viral replication. We describe here a cellular assay in the yeast *Saccharomyces cerevisiae* for the identification of small molecule inhibitors of HCMV protease by conditional growth in selective medium. In this system, the protease cleavage sequence is inserted into the N-(5'-phosphoribosyl)-anthranilate isomerase (Trp1p), a yeast protein essential for cell proliferation in the absence of tryptophan. Co-expression of HCMV protease with the engineered Trp1p substrate in yeast cells results in site-specific cleavage and functional inactivation of the Trp1p enzyme, thereby leading to an arrest of cell proliferation. This growth arrest can be suppressed by the addition of validated HCMV protease inhibitors. The growth selection system presented here provides the basis for a high-throughput screen to identify HCMV protease inhibitors that are active in eukaryotic cells.

Novel Yeast Cell-Based Assay To Screen for Inhibitors of Human Cytomegalovirus Protease in a High-Throughput Format

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The protease encoded by the human cytomegalovirus (HCMV) is an attractive target for antiviral drug development because of its essential function in viral replication. We describe here a cellular assay in the yeast *Saccharomyces cerevisiae* for the identification of small molecule inhibitors of HCMV protease by conditional growth in selective medium. In this system, the protease cleavage sequence is inserted into the *N*-(5'-phosphoribosyl)anthranilate isomerase (Trp1p), a yeast protein essential for cell proliferation in the absence of tryptophan. Coexpression of HCMV protease with the engineered Trp1p substrate in yeast cells results in site-specific cleavage and functional inactivation of the Trp1p enzyme, thereby leading to an arrest of cell proliferation. This growth arrest can be suppressed by the addition of validated HCMV protease inhibitors. The growth selection system presented here provides the basis for a high-throughput screen to identify HCMV protease inhibitors that are active in eukaryotic cells.

Herpesviruses are widely present in nature and afflict many species throughout the animal kingdom (14). The most frequent human infection is caused by the human cytomegalovirus (HCMV), affecting up to 80% of the general population. This highly prevalent member of the herpesvirus family is responsible for opportunistic infections in immunocompromised individuals, notably AIDS patients and organ transplant recipients (for reviews, see references 9 and 29). Antiviral agents currently licensed for the treatment of HCMV infections include ganciclovir and its orally bioavailable prodrug valganciclovir as well as foscarnet, cidofovir, and fomivirsen (9). All these drugs are nucleoside analogues that ultimately target, either directly or indirectly, the viral DNA polymerase. The only exception is fomivirsen, an antisense oligonucleotide approved for HCMV retinitis that blocks translation of HCMV immediate-early mRNA. Unfortunately for the patients, the clinical usefulness of these drugs is limited: they exhibit toxic side effects, including bone marrow toxicity and nephrotoxicity, and they need to be injected either intravenously or intraocularly (9). Moreover, HCMV strains with reduced susceptibility resulting from chronic antiviral treatment are becoming more and more frequent. Thus, improved alternative drugs with novel mechanisms of action are needed for treating HCMV infections.

Herpesviruses encode a serine protease that cleaves the assembly protein precursor, a major component of the intermediate capsid (reviewed in reference 23). Studies with herpes simplex virus type 1 protease, a close homologue of HCMV protease, showed that viral DNA cannot be packaged into virions without this cleavage, resulting in an empty nucleocapsid (17, 32). HCMV protease is expressed as a 708-amino-acid precursor encoded by the UL80 open reading frame. Autocatalytic cleavage is observed at two consensus sequences called maturational (M) and release (R) sites (23). M-site cleavage

removes a 6-kDa C-terminal tail, which mediates interaction with the major capsid protein at the earliest stage of capsid assembly. Cleavage at the R-site releases the N-terminal 28-kDa proteolytic domain. Enzymatic *in vitro* assays with short peptide substrates demonstrated a 10-fold increase in the k_{cat}/K_m value for an M-site peptide substrate over that for an R-site substrate (37).

Four different groups elucidated the X-ray crystallographic structure of HCMV protease in 1996 (8, 33, 36, 38). These studies have unequivocally confirmed the status of this enzyme as a serine protease with a unique catalytic triad. Instead of the His-Ser-Asp/Glu residues present in the active site of classical serine proteases, HCMV protease contains an unusual His residue at the third position. Moreover, only the dimeric form is active, which sets it further apart from other serine proteases (28). The two active sites are well separated on opposite faces of the dimer and act in an independent manner (4). The fact that the protease is essential for the propagation of the virus and that it markedly differs from mammalian serine proteases makes this enzyme an attractive therapeutic target.

Though peptidomimetic drug design delivered a number of HCMV protease inhibitors with good *in vitro* potency in the submicromolar range, e.g., transactams (5) and 2-substituted benzoxazinones (1), their activity in cell culture remained limited. In addition, high-throughput screening (HTS) campaigns in the industry, based on enzymatic *in vitro* assays, did not lead to the identification of hits with good activity in cell culture assays. Remarkably, very few reports describe the investigation of HCMV protease activity in a cellular environment (26, 41), and to our knowledge, no cell-based HTS has been performed so far. More efforts should be invested in such cellular assays, since they not only allow for screening in a natural cellular environment but also directly exclude compounds that are unstable or toxic or that cannot penetrate biological membranes. Thus, appropriate cell-based assays can accelerate the drug discovery process (for reviews, see references 2 and 3).

In an effort to select for HCMV protease inhibitors in an *in vivo* environment, we have established a target-specific HTS

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system in *Saccharomyces cerevisiae* that detects protease activity by conditional growth in selective medium. In a proof-of-principle experiment, we show that the application of known HCMV protease inhibitors results in a concentration-dependent stimulation of cell proliferation.

MATERIALS AND METHODS

Yeast strains. The genes encoding the three major ABC transporter proteins Pdr5p, Snq2p, and Yor1p were deleted in the *S. cerevisiae* JPY5 strain (*MAT α* *ura3-52 his3 Δ 200 leu2 Δ 1 trp1 Δ 63 lys2 Δ 385*) to generate the RLY07 strain (*MAT α* *ura3-52 his3 Δ 200 leu2 Δ 1 trp1 Δ 63 lys2 Δ 385 pdr5 Δ snq2 Δ yor1 Δ*). All recombinant proteins described in this study were expressed in RLY07 cells.

Recombinant plasmids. The *S. cerevisiae* *TRP1* gene was amplified by PCR from the plasmid YCplac22 (20). All *TRP1-M* constructs generated hereupon were subcloned via unique XbaI and SalI restriction sites in the CEN4-ARS1 plasmid pMH4. pMH4 carries a *LEU2* auxotrophic marker and expresses subcloned *TRP1-M* constructs under the control of a 5' truncated version of the *ADH* promoter and the *GAL11* terminator. The HCMV protease cleavage sequence GGVVNA↓SCRLAGG, derived from the M site of the 75-kDa protease precursor, was flanked with unique NcoI and NotI sites and inserted by PCR directly to the C-terminal end of amino acid residues 49, 102, 132, 165, and 194 of the Trp1 protein. This generated a plasmid series encoding the recombinant proteins Trp1^{49-M}, Trp1^{102-M}, Trp1^{132-M}, Trp1^{165-M}, and Trp1^{194-M}. In order to generate Trp1^{194-Me} (for Trp1^{194-M} elongated), the 13-amino-acid cleavage sequence was replaced (via NcoI and NotI sites) by an extended M-type sequence consisting of 39 amino acids (see Fig. 3). For Western blotting, hemagglutinin (HA) epitope tags were added to both termini of the Trp1^{194-Me} construct. The HCMV protease gene encoding amino acids 1 to 256 of the 708-amino-acid protease precursor was obtained by PCR from HCMV-infected MRC5 human cells and subcloned via unique XbaI and NotI sites in pMH51, a CEN4-ARS1 plasmid with a *URA3* selection marker and a full-length (100%) *GAL1* promoter. In the experiment for which results are shown in Fig. 4, the HCMV protease gene was subcloned in a plasmid series with distinct *GAL1* promoters that express the protease with 71%, 46%, and 16% protein production levels relative to the original 100% *GAL1* promoter. The *GAL1* promoter contains four different pseudopalindromic binding sites for the transcription factor Gal4p. Liang et al. have shown that modifications in the number and type of Gal4p binding sites modulate transcription of a downstream cloned reporter gene (27). We have differentially deleted those binding modules and validated promoter strength with a downstream cloned *lacZ* reporter gene (above 71%, 46%, and 16% protein production levels).

Yeast media and transformation. All media were prepared according to Burke et al. (7). Transformation of yeast cells was performed following the lithium acetate method (19).

Spotting assay. RLY07 cells transformed with the different *TRP1-M* constructs were inoculated in 3 ml synthetic dropout (SD) glucose medium lacking Leu (–Leu) and grown to an optical density at 595 nm (OD₅₉₅) of 1 to reach an exponential growth phase. Cultures were hereupon washed with 5 ml H₂O, resuspended in –Leu –Trp SD glucose medium and diluted to 10⁶ cells/ml. Ten microliters of serially diluted culture was spotted on nonselective (–Leu) and selective (–Leu –Trp) glucose SD plates and incubated at 30°C for 3 days.

Liquid growth assays. RLY07 cells transformed with the different *TRP1-M* constructs and the HCMV protease gene were inoculated in 3 ml –Leu –Ura galactose SD medium and grown at 30°C to an OD₅₉₅ of 1. They were then washed with 5 ml H₂O and resuspended in –Leu –Ura –Trp galactose SD medium supplemented with 10% glycerol (growth selection medium). It is likely to assume that glycerol promotes HCMV protease dimerization and subsequent proteolytic activity, as it has been shown in vitro assays (28). In yeast, glycerol is taken up by a proton symport mechanism (13). Liquid growth assays were started at an inoculation OD₅₉₅ of 0.01 in growth selection medium in 96-well microtiter plates in a volume of 150 μ l per well. Cultures were incubated at 30°C without shaking. At defined time points (as specified in figure legends), plates were shaken at 1,000 rpm for 1 min to completely resuspend cells, and cell density was measured at 595 nm on a Tecan GENios reader (Männedorf, Switzerland). HCMV protease inhibitors BI31 and BI36 (Boehringer Ingelheim, Québec) were dissolved in dimethyl sulfoxide and added to the cultures directly at the beginning of the growth assay; final dimethyl sulfoxide concentration was 1%.

Western blot analysis. Yeast whole-cell extracts were prepared as described by Burke et al. (7). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blot analysis was performed according to

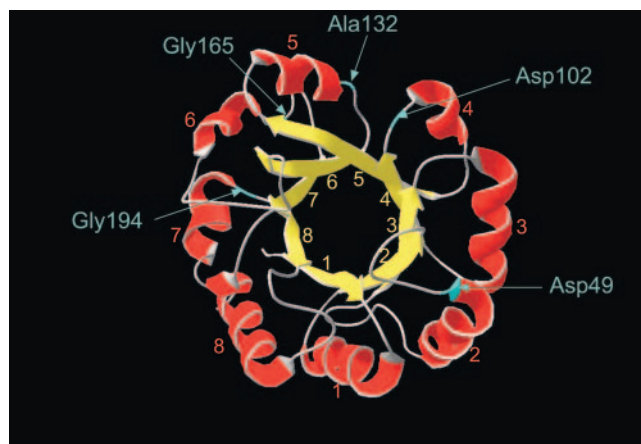


FIG. 1. Modeled structure of the *S. cerevisiae* *N*-(5'-phosphoribosyl)anthranilate isomerase Trp1p. Residues preceding the inserted M-cleavage sequences are indicated in blue.

standard procedures. An anti-HA monoclonal antibody from Sigma (clone 3F10) was used at a concentration of 30 ng/ml to detect expression of Trp1^{194-Me}.

RESULTS

A cell-based system to detect protease activity. In the present work, we describe a cell growth selection system in the yeast *S. cerevisiae* that (i) can detect and characterize HCMV protease activity and (ii) is applicable to screen for HCMV protease inhibitors in a high-throughput format. To establish this system, we inserted an HCMV protease cleavage site into a yeast protein that is conditionally essential for cell proliferation. This protein was chosen from the auxotrophic growth markers, considering that most laboratory strains are already deleted for these genes and that therefore, such a system could be applied in almost any genetic background. Since the *N*-(5'-phosphoribosyl)anthranilate isomerase (Trp1p) enzyme has been intensively studied (10, 11, 22, 24) and its three-dimensional structure has been determined for different organisms, we have chosen this member of the tryptophan synthesis pathway as a substrate for HCMV protease.

Thus, in our setup, such an engineered Trp1 enzyme should be cleaved and subsequently inactivated by HCMV protease, resulting in a reduced growth in selective medium lacking tryptophan.

Insertion of the HCMV protease cleavage sequence at five different locations in Trp1p. Two conditions are critical for appropriate operation of the above-described system. (i) Insertion of the HCMV protease cleavage sequence must preserve Trp1 function in the tryptophan biosynthesis pathway. (ii) On the other hand, cleavage at the inserted sequence must result in functional inactivation of the Trp1 enzyme.

The Trp1 enzyme is a member of the prominent class of proteins that fold into a (β/α)₈ barrel, which is the most commonly occurring fold among enzymes (12) (Fig. 1). The *S. cerevisiae* Trp1p structure has not yet been determined, but amino acid sequence alignments with the *N*-(5'-phosphoribosyl)anthranilate isomerase from *Escherichia coli* and *Thermotoga maritima* provide us with a reliable model. *S. cerevisiae* Trp1p shares 28% identical amino acids with *E. coli* Trp1p and

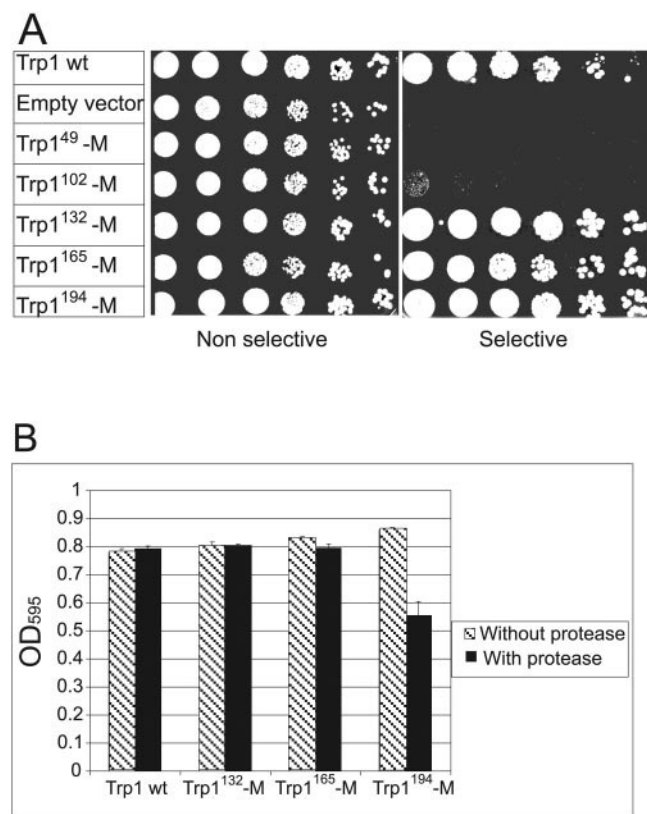


FIG. 2. Functional tests of the five engineered Trp1p constructs. (A) Serial dilutions of RLY07 cells transformed with wild-type (wt) Trp1p (positive control), empty vector (negative control), Trp1⁴⁹-M, Trp1¹⁰²-M, Trp1¹³²-M, Trp1¹⁶⁵-M, or Trp1¹⁹⁴-M were spotted on selective -Trp plates and incubated 3 days at 30°C. (B) Wild-type Trp1p, Trp1¹³²-M, Trp1¹⁶⁵-M, and Trp1¹⁹⁴-M constructs, cotransformed in RLY07 either with an empty vector (dashed bars) or with active HCMV protease (black bars) were grown in selective -Trp medium for 35 h; cell density was measured at 595 nm. Data are expressed as means from three independent experiments \pm standard deviations.

33% with *T. maritima* Trp1p. Alignment and modeling for *S. cerevisiae* Trp1p was performed with the SWISS-MODEL protein modeling server (<http://swissmodel.expasy.org/repository/>) (21, 35).

To determine suitable sites for insertion of the cleavage sequence, several constructs were designed. Since turn sequences are in general highly mutable in (β/α)₈ barrels, five insertion sites were chosen that are located in such turns between an α -helix and a β -sheet, after amino acids Asp⁴⁹, Asp¹⁰², Ala¹³², Gly¹⁶⁵, and Gly¹⁹⁴ (Fig. 1A). Respective constructs will therefore be referred to as Trp1⁴⁹-M, Trp1¹⁰²-M, Trp1¹³²-M, Trp1¹⁶⁵-M, and Trp1¹⁹⁴-M. The inserted sequence consists of 13 amino acids derived from the M site of the 75-kDa protease precursor (see Fig. 3A, sequence a).

In order to evaluate the functionality of the Trp1⁴⁹-M, Trp1¹⁰²-M, Trp1¹³²-M, Trp1¹⁶⁵-M, and Trp1¹⁹⁴-M fusion proteins, a spotting assay was performed (Fig. 2A). RLY07 cells were transformed with these constructs, and their ability to proliferate was assessed on selective plates lacking tryptophan. For Trp1¹³²-M-, Trp1¹⁶⁵-M-, and Trp1¹⁹⁴-M-expressing cells, growth was indistinguishable from that of cells expressing wild-

type Trp1p, indicating that M-site insertion did not interfere with the functionality of the enzyme. In contrast, Trp1⁴⁹-M and Trp1¹⁰²-M constructs produced nonfunctional enzymes, as demonstrated by likewise-transformed cells unable to grow on selective plates (Fig. 2A).

Sequence-specific cleavage of Trp1¹⁹⁴-M by HCMV protease in yeast. We next investigated whether the three functional Trp1-M proteins are cleaved and inactivated by HCMV protease. Trp1¹³²-M, Trp1¹⁶⁵-M, and Trp1¹⁹⁴-M were coexpressed with the viral protease in the RLY07 strain, and cell proliferation was assayed by measuring OD₅₉₅ of transformed cells cultured in growth selection medium (Fig. 2B). After 36 h, cells coexpressing Trp1¹⁹⁴-M with the protease exhibited a growth reduction of 35% compared to control cells that contained an empty vector instead of the protease-expressing plasmid, indicating that cleavage between helix α 7 and strand β 8 reduces activity of the Trp1 enzyme. Interestingly, this region is situated close to the phosphate binding site of the anthranilate substrate. Crystallographic studies have shown that the two neighboring loops between β 7 and α 7 and between β 8 and α 8 are important for binding of the phosphate ion (40). Cleavage in this region might cause structural changes, thus preventing binding of the substrate. As opposed to Trp1¹⁹⁴-M cells, Trp1¹³²-M- as well as Trp1¹⁶⁵-M-expressing cells did not show growth reduction upon coexpressing HCMV protease (Fig. 2B). Thus, the latter two engineered Trp1 substrates were either not cleaved or, alternatively, cleavage occurred but the separated fragments still formed an active enzyme.

To improve cleavage frequency at the M site of the Trp1¹⁹⁴-M substrate, the 13-amino-acid target sequence was replaced by a longer sequence consisting of 39 amino acids (Fig. 3A, sequence b). Cells expressing the modified Trp1¹⁹⁴-Me together with the active protease showed 85% reduction of cell proliferation compared to the 35% reduction with the original, shorter cleavage site (Fig. 3B). This indicates that the extended recognition site is more efficiently cleaved by the protease.

The HCMV protease has been shown to hydrolyze both the M site and R site of the protease precursor between an alanine and a serine (6). To demonstrate site-specific cleavage of the Trp1¹⁹⁴-Me substrate, we replaced the alanine of the scissile bond with glutamic acid (Fig. 3A, sequence c), a mutation known to prevent cleavage (39). As expected, the proliferation of cells coexpressing the Trp1¹⁹⁴-Me(A \rightarrow E) mutant with HCMV protease was comparable to the proliferation of cells expressing Trp1¹⁹⁴-Me alone, providing evidence that the Trp1¹⁹⁴-Me substrate is cleaved in a sequence-specific manner at the scissile bond (Fig. 3B). A further control experiment was performed by using an enzymatically inactive version of HCMV protease harboring the S132A mutation at the active-site serine (8). As shown in Fig. 3B, this mutant was not able to cleave the Trp1¹⁹⁴-Me substrate.

To demonstrate actual cleavage of the substrate in yeast, Trp1¹⁹⁴-Me was tagged with an HA epitope and analyzed by Western blotting. The full-length substrate migrates at the level of 33-kDa proteins in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis assay (Fig. 3C, lane 1). Coexpression of active HCMV protease (Fig. 3C, lane 2) causes the disappearance of the full-length substrate upon Western blot analysis of the respective yeast extracts. However, no cleaved

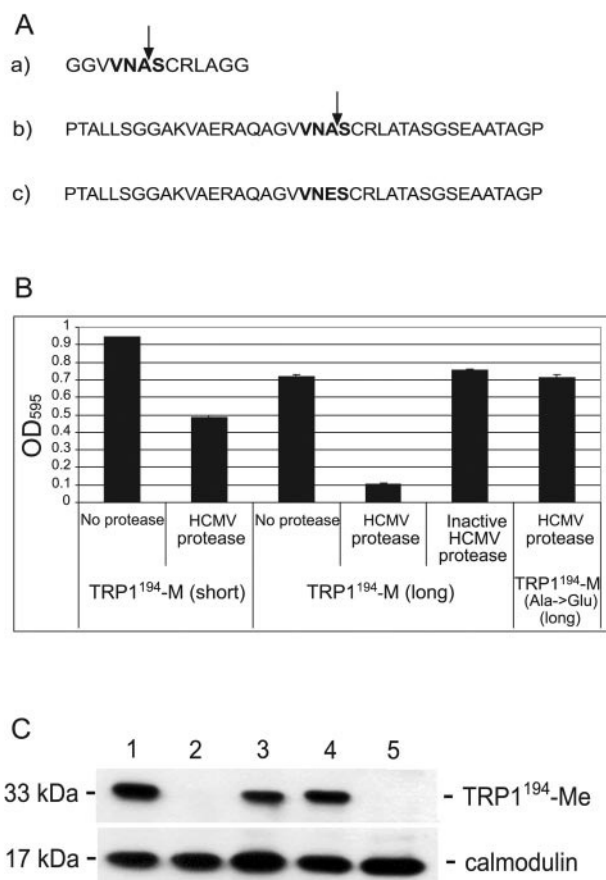


FIG. 3. Sequence-specific cleavage of the Trp1¹⁹⁴-Me substrate by the HCMV protease. (A) HCMV protease cleavage sequences inserted C terminal to G194 of Trp1p. a) 13 residues (short) with the M site; b) 39 residues (long) with the M site; c) long M-site sequence with the point mutation A→E at the scissile bond. (B) RLY07 cells were cotransformed with the different Trp1¹⁹⁴-M substrates and a plasmid expressing either active or inactive (active-site point mutation S132A) HCMV protease, as indicated. Cells were grown in selective -Trp medium, and OD₅₉₅ was measured after 38 h. Data are expressed as means from three independent experiments ± standard deviations. (C) Western blot analysis of Trp1¹⁹⁴-Me cleavage by HCMV protease. The uncleaved 33-kDa Trp1¹⁹⁴-Me substrate, which carries HA tags at both termini, is detected by immunoblotting with an anti-HA antibody. Protein extracts were made from transformed RLY07 cells expressing Trp1¹⁹⁴-M (lane 1), Trp1¹⁹⁴-M and active HCMV protease (lane 2), Trp1¹⁹⁴-M and inactive HCMV protease (lane 3), Trp1¹⁹⁴-Me(A→E) and active HCMV protease (lane 4), and empty vectors (negative control) (lane 5). The calmodulin antibody was used as an internal control for protein amount.

fragments could be detected. We suppose that they are either degraded or below the detection threshold of the Western blot. Lane 3 of Fig. 3C shows that the inactive viral protease does not cleave Trp1¹⁹⁴-Me since the full-length substrate band does not disappear. Lane 4 shows that the active protease has no effect on the point-mutated Trp1¹⁹⁴-Me(A→E) substrate.

Taken together, the above-described experiments demonstrate that the Trp1¹⁹⁴-Me substrate is cleaved in a sequence-specific manner by HCMV protease and that this cleavage results in a slow-growth phenotype.

Modulation of HCMV protease expression level correlates with distinct changes in cell proliferation. A prerequisite for a

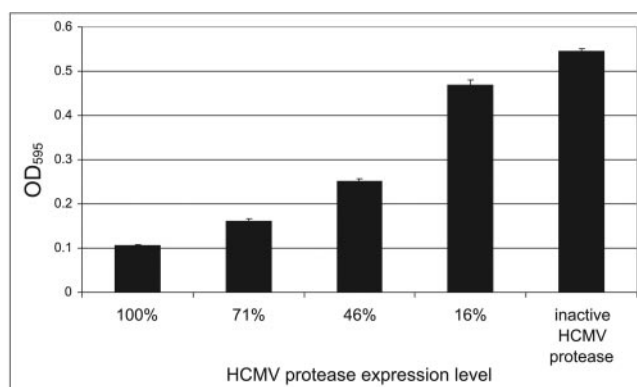


FIG. 4. A gradual decrease of HCMV protease expression correlates with distinct stimulation of cell proliferation. In the first four columns, RLY07 cells were cotransformed with Trp1¹⁹⁴-Me and with active HCMV protease expressed from a *GAL1* promoter series (see Materials and Methods for detailed information). The last column shows cell growth mediated by the inactive protease. Cell densities were monitored after 30 h by measuring OD₅₉₅. Data are expressed as means from three independent experiments ± standard deviations.

successful HTS is that even only partial inhibition of the protease can be detected in the assay. We were therefore interested to find out whether different concentrations of intracellular active protease result in distinguishable and selectable levels of growth rate on the HTS read-out. In the experiments described above, HCMV protease was expressed from the full-length *GAL1* promoter (100% activity). To express the protease at distinct lower levels, thus mimicking partial protease inhibition by a small molecule in a screening experiment, the protease was subcloned on a *GAL1* vector series, with discrete protein production levels of 71%, 46%, and 16% compared to the original 100% promoter. These plasmids were cotransformed with the Trp1¹⁹⁴-Me substrate in RLY07 cells, and cell growth was measured after 36 h at OD₅₉₅. Figure 4 shows that a gradual decrease of promoter strength, and thus of intracellular protease activity, is inversely proportional to cell proliferation. For example, a reduction of 29% of protease expression (from the 100% promoter to the 71% promoter) resulted in a 53% stimulation of cell proliferation. Likewise, a reduction of 54% of protease expression caused a stimulation of 138%. Therefore, even weak inhibitors that cause only a partial reduction of HCMV protease activity should be detectable in our system.

Two validated HCMV protease inhibitors stimulate cell proliferation in a screening assay format. The assay described above was developed to identify cell-permeable small molecule inhibitors of HCMV protease in high-throughput screening experiments. We have already shown that the system is able to distinguish different levels of intracellular protease activity. In order to assess sensitivity of the selection system towards small molecules, the two validated HCMV protease inhibitors BI31 and BI36 of Boehringer Ingelheim were applied (42). BI31 and BI36 show 50% inhibitory concentration values of 1.7 and 0.5 μM in an enzymatic in vitro assay and inhibit viral replication in cell culture with 50% effective concentration (EC₅₀) values of 95 and 78 μM, respectively (42). Both compounds are based on a 4-thioalkyl β-lactam scaffold. Lactam derivatives have

initially been published as inhibitors of classical serine proteases, such as human leukocyte elastase (15). Further development of such scaffolds by rational design delivered inhibitors specific for HCMV protease (42).

RLY07 cells coexpressing the Trp1¹⁹⁴-Me substrate and HCMV protease were incubated with various concentrations of BI31 and BI36 in a 96-well microtiter plate and cultivated under selective conditions at 30°C for 44 and 28 h, respectively. Figure 5A shows that increasing concentrations of both BI31 and BI36 in cells expressing the active protease correlate with increasing cell proliferation. The calculated EC₅₀ of 31 μ M for BI36 suggests that the yeast-based assay exhibits the same sensitivity as that of a mammalian cellular assay, at least for this class of compounds (42). At BI36 concentrations of >100 μ M, HCMV protease was strongly inhibited, as evidenced by proliferation rates similar to those of cells expressing the inactive protease. Though BI31 also restored cell growth in a concentration-dependent manner, stimulation was not as pronounced as with BI36 due to cellular toxicity of this compound. This toxicity is also shown by the fact that increasing concentrations of BI31 in RLY07 cells expressing the inactive (active-site mutated S132A) HCMV protease caused a gradual decrease of cell proliferation. Importantly, despite this toxicity, BI31 still stimulated growth of cells expressing the active protease. For example, at 50 μ M cell density is multiplied by a factor 4 despite 25% toxicity. This suggests that in an HTS, compounds will be scored as positives even if they exert some intrinsic toxicity.

Inhibition of HCMV protease activity in yeast by BI36 was also addressed by Western blot analysis (Fig. 5B). As already shown in Fig. 3C, a 33-kDa band corresponding to the un-cleaved full-length Trp1¹⁹⁴-Me substrate can be detected in cells coexpressing the Trp substrate with the inactive (S132A) protease. In cells cotransformed with the active protease instead of the inactive version, disappearance of the 33-kDa band can be observed. Importantly, the addition of 100 μ M BI36 to the growth selection medium of those cells almost completely prevented cleavage of the substrate (Fig. 5B). When using lower concentrations of BI36 (10 μ M), the full-length substrate band disappears again, suggesting that at concentrations well below the calculated EC₅₀ of 31 μ M in yeast, only a small percentage of protease activity is inhibited.

In summary, we have shown that two well-validated active-site inhibitors of HCMV protease cause a concentration-dependent stimulation of cell growth in our yeast assay. Importantly, sensitivity of the yeast system towards these two compounds is comparable to the sensitivity of a mammalian cell-based assay. The calculated Z' factor values of 0.85 for 50 μ M BI36 and 0.75 for 50 μ M BI31 clearly demonstrate the suitability of this bioassay for high-throughput applications.

DISCUSSION

The frequent emergence of cytomegalovirus strains resistant against classical antiviral agents, which all directly or indirectly target viral DNA polymerase, spurred the search for alternative therapeutic targets of the virus (reviewed in reference 29). The cytomegalovirus protease, or assemblin, has gained much attention as a target due to the unique serine protease fold and catalytic triad that is expected to facilitate the design of virus-

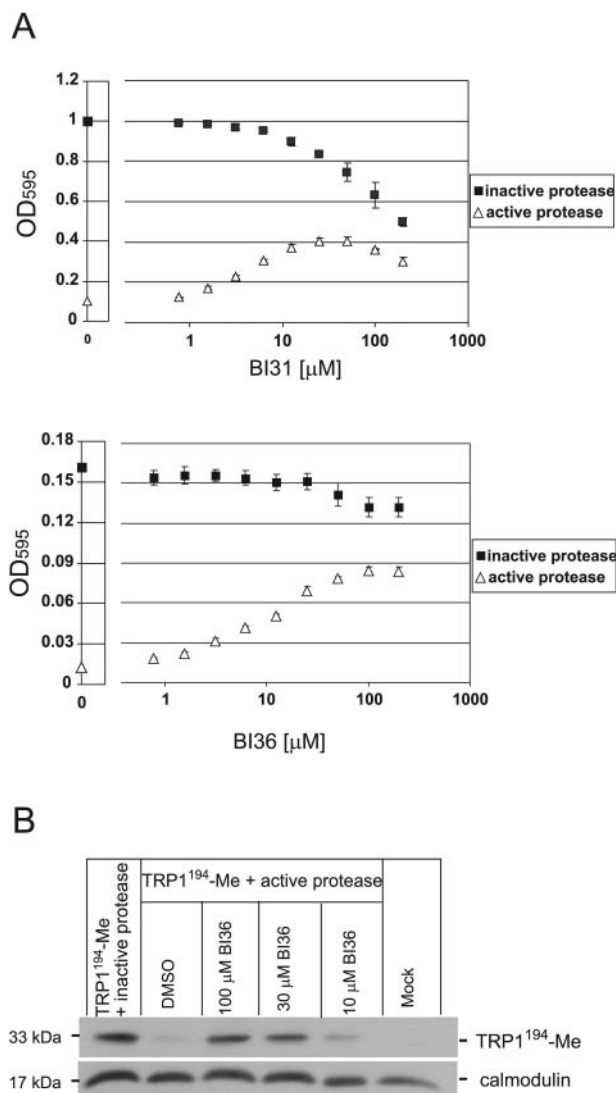


FIG. 5. Validated HCMV protease inhibitors BI31 (upper panel) and BI36 (lower panel) stimulate cell proliferation in a concentration-dependent manner. (A) RLY07 cells expressing Trp1¹⁹⁴-Me and active HCMV protease (triangles) or Trp1¹⁹⁴-Me and inactive protease (squares) were cultivated either in the absence (0) or presence of increasing concentrations of BI31 and BI36. Cells were grown in 150- μ l cultures in a 96-well microplate for 44 h (BI31) and 28 h (BI36) before OD₅₉₅ was measured. Data are expressed as means from three independent experiments \pm standard deviations. (B) Western blot analysis with HA antibody showing that application of BI36 prevents cleavage of the Trp1¹⁹⁴-Me substrate by HCMV protease. RLY07 cells were transformed with Trp1¹⁹⁴-Me and inactive protease (positive control), Trp1¹⁹⁴-Me and active protease, or Trp1¹⁹⁴-Me with active protease in the presence of distinct concentrations of BI36, as indicated. RLY07 cells containing two empty plasmids were used as a negative control. The calmodulin antibody served as an internal control for protein amount. DMSO, dimethyl sulfoxide.

specific inhibitors. Though past screening campaigns have culminated in the identification of inhibitors with nanomolar activities in enzymatic in vitro assays, their activity in cell-based assays was limited in most cases. Examples include benzoxazinones (16), lactams (5, 18), and the highly toxic enedione derivatives (30). To our knowledge, no cell-based HTS on

herpesviral proteases has so far been performed successfully. Compared to in vitro assays, cellular systems allow for the screening of the therapeutic target in its natural configuration within the cell. In addition, they select for compounds that are stable within a metabolic environment, that are able to penetrate biological membranes, and that show no or only limited toxic effects on the cell (for reviews, see references 2 and 3). Therefore, appropriate cellular systems potentially accelerate the drug discovery process.

We have established a cellular assay in the yeast *S. cerevisiae* that detects HCMV protease activity and that enables screening for small molecule protease inhibitors with a positive growth read-out. Yeast provides a eukaryotic environment in a single cell with a high degree of conserved basic molecular mechanisms with mammalian cells. In addition, yeast is inexpensive to propagate and can be easily genetically engineered.

For establishing the growth selection system described here, a 39-amino-acid HCMV protease cleavage sequence derived from the M site of the 75-kDa protease precursor was inserted into the phosphoribosylanthranilate isomerase, a yeast enzyme involved in the biosynthesis of tryptophan and therefore essential for cell proliferation in the absence of tryptophan. Coexpression of HCMV protease with this engineered substrate caused sequence-specific cleavage at the M site, resulting in growth arrest (Fig. 3B). Importantly, the addition of validated cell-active β -lactam inhibitors restored cell proliferation in a concentration-dependent manner (Fig. 5B). Since yeast encodes a pleiotrophic drug resistance network with a variety of ABC transporters that efficiently clear small molecules within the cell, we sensitized the strain by deleting the three major drug efflux pumps Pdr5p, Snq2p, and Yor1p. In such a setup, the yeast system shows a sensitivity towards small molecules similar to that shown in a mammalian cellular assay, at least for the β -lactam inhibitors tested, with a calculated EC_{50} of 31 μ M in the yeast assay versus 78 μ M in a mammalian plaque reduction assay (42).

HCMV protease has been shown to be active as a dimer (28). Remarkably, enzymatic in vitro assays require high concentrations of dimerization-promoting agents, such as anti-chaotropic salts and glycerol, that dramatically increase the k_{cat}/K_m value to obtain reasonable turnover rates of the enzyme (25, 28). The fact that in our cellular assay a gradual increase of glycerol concentration correlates inversely with cell proliferation (data not shown) underscores the hypothesis that HCMV protease is also a dimer in yeast. This is confirmed by the observation that the point mutation L221A completely inactivates HCMV protease in yeast (data not shown). It has been demonstrated for Kaposi's sarcoma-associated herpesvirus protease that mutating the homologous residue L196 to an alanine abrogates protease activity by preventing dimerization (31).

It is a subject of debate whether the 29-kDa assemblin or the 75-kDa protease precursor is the physiologically relevant protease responsible for capsid maturation and therefore the appropriate therapeutic target. While all enzymatic HTS has been performed with the 29-kDa domain, Robertson et al. suggested the 75-kDa precursor to be the "real" target. Indeed, transcomplementation studies with mutant herpes simplex virus type 1 viruses have shown that the proteolytic activity essential for capsid maturation is provided by the full-length

precursor protein (34). We coexpressed Trp1¹⁹⁴-Me with the 75-kDa protease precursor and observed a minor but statistically significant reduction in cell growth (data not shown). We are currently investigating whether this proteolytic activity is derived from the protease precursor per se or whether the 29-kDa assemblin, generated upon autocatalytic cleavage, is responsible for cleavage of the Trp1 substrate.

Applicability of the above-described Trp1p-based yeast assay is not limited to HCMV protease and can be exploited for other proteases. We have replaced the M site of the Trp1¹⁹⁴-Me substrate with a 13-amino-acid cleavage sequence of the coxsackievirus 3C cysteine protease. Coexpression of the likewise-engineered Trp1 substrate with the 3C protease resulted in an arrest of cell proliferation (data not shown). In a proof-of-concept study, we have used the Trp1-M system as a validation tool for hits identified in a high-throughput screen of a 15,000-chemical-compound library. With this approach, we have identified a number of compounds that also show activity in a mammalian plaque reduction assay in the low-micromolar range (unpublished data).

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REFERENCES

1. Abood, N. A., L. A. Schretzman, D. L. Flynn, K. A. Houseman, A. J. Wittwer, V. M. Dilworth, P. J. Hippenmeyer, and B. C. Holwerda. 1997. Inhibition of human cytomegalovirus protease by benzoxazinones and evidence of antiviral activity in cell culture. *Bioorg. Med. Chem. Lett.* 7:2105–2108.
2. Barberis, A. 2002. Cell-based high-throughput screens for drug discovery. *Eur. BioPharm. Rev.* 2002:93–96.
3. Barberis, A., T. Gunde, C. Berset, S. Audetat, and U. Lüthi. 2005. Yeast as a screening tool. *Drug Discov. Today* 2:187–192.
4. Batra, R. 2001. Molecular mechanism for dimerization to regulate the catalytic activity of human cytomegalovirus protease. *Nat. Struct. Biol.* 8:810–817.
5. Borthwick, A. D. 2005. Design of transactant HCMV protease inhibitors as potent antivirals. *Med. Res. Rev.* 25:427–452.
6. Burck, P. J., D. H. Berg, T. P. Luk, L. M. Sassmannshausen, M. Wakulchik, D. P. Smith, H. M. Hsiung, G. W. Becker, W. Gibson, and E. C. Villarreal. 1994. Human cytomegalovirus maturational proteinase: expression in *Escherichia coli*, purification, and enzymatic characterization by using peptide substrate mimics of natural cleavage sites. *J. Virol.* 68:2937–2946.
7. Burke, D., D. Dawson, and T. Stearns. 2000. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
8. Chen, P., H. Tsuge, R. J. Almassy, C. L. Gribskov, S. Katoh, D. L. Vanderpool, S. A. Margosiak, C. Pinko, D. A. Matthews, and C. C. Kan. 1996. Structure of the human cytomegalovirus protease catalytic domain reveals a novel serine protease fold and catalytic triad. *Cell* 86:835–843.
9. De Clercq, E. 2003. New inhibitors of human cytomegalovirus (HCMV) on the horizon. *J. Antimicrob. Chemother.* 51:1079–1083.
10. Eder, J., and K. Kirschner. 1992. Stable substructures of eightfold beta alpha-barrel proteins: fragment complementation of phosphoribosylanthranilate isomerase. *Biochemistry* 31:3617–3625.
11. Eder, J., and M. Wilmanns. 1992. Protein engineering of a disulfide bond in a beta/alpha-barrel protein. *Biochemistry* 31:4437–4444.
12. Farber, G. K., and G. A. Petsko. 1990. The evolution of alpha/beta barrel enzymes. *Trends Biochem. Sci.* 15:228–234.
13. Ferreira, C., F. van Voorst, A. Martins, L. Neves, R. Oliveira, M. C. Kieland-Brandt, C. Lucas, and A. Brandt. 2005. A member of the sugar transporter family, Stt1p is the glycerol/H⁺ symporter in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 16:2068–2076.
14. Fields, B. N., D. M. Knipe, and P. M. Howley. 1996. Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
15. Finke, P. E., S. K. Shah, D. S. Fletcher, B. M. Ashe, K. A. Brause, G. O. Chandler, P. S. Dellea, K. M. Hand, A. L. Maycock, D. G. Osinga, et al. 1995. Orally active beta-lactam inhibitors of human leukocyte elastase. 3. Stereospecific synthesis and structure-activity relationships for 3,3-dialkylazetidin-2-ones. *J. Med. Chem.* 38:2449–2462.
16. Flynn, D. L., N. A. Abood, and B. C. Holwerda. 1997. Recent advances in antiviral research: identification of inhibitors of the herpesvirus proteases. *Curr. Opin. Chem. Biol.* 1:190–196.

17. Gao, M., L. Matusick-Kumar, W. Hurlburt, S. F. DiTusa, W. W. Newcomb, J. C. Brown, P. J. McCann III, I. Deckman, and R. J. Colonno. 1994. The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth. *J. Virol.* **68**:3702–3712.
18. Gerona-Navarro, G., M. J. Perez de Vega, M. T. Garcia-Lopez, G. Andrei, R. Snoeck, J. Balzarini, E. De Clercq, and R. Gonzalez-Muniz. 2004. Synthesis and anti-HCMV activity of 1-acyl-beta-lactams and 1-acylazetidines derived from phenylalanine. *Bioorg. Med. Chem. Lett.* **14**:2253–2256.
19. Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* **20**:1425.
20. Gietz, R. D., and A. Sugino. 1988. New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**:527–534.
21. Guex, N., and M. C. Peitsch. 1997. SWISS-MODEL and the Swiss-Pdb-Viewer: an environment for comparative protein modeling. *Electrophoresis* **18**:2714–2723.
22. Hennig, M., R. Sterner, K. Kirschner, and J. N. Jansonius. 1997. Crystal structure at 2.0 Å resolution of phosphoribosyl anthranilate isomerase from the hyperthermophile *Thermotoga maritima*: possible determinants of protein stability. *Biochemistry* **36**:6009–6016.
23. Holwerda, B. C. 1997. Herpesvirus proteases: targets for novel antiviral drugs. *Antivir. Res.* **35**:1–21.
24. Hommel, U., M. Eberhard, and K. Kirschner. 1995. Phosphoribosyl anthranilate isomerase catalyzes a reversible amadori reaction. *Biochemistry* **34**:5429–5439.
25. Khayat, R., R. Batra, G. A. Beberitz, M. W. Olson, and L. Tong. 2004. Characterization of the monomer-dimer equilibrium of human cytomegalovirus protease by kinetic methods. *Biochemistry* **43**:316–322.
26. Lawler, J. F., Jr., and S. H. Snyder. 1999. Viral protease assay based on GAL4 inactivation is applicable to high-throughput screening in mammalian cells. *Anal. Biochem.* **269**:133–138.
27. Liang, S. D., R. Marmorstein, S. C. Harrison, and M. Ptashine. 1996. DNA sequence preferences of GAL4 and PPR1: how a subset of Zn²⁺ Cys₆ binuclear cluster proteins recognizes DNA. *Mol. Cell. Biol.* **16**:3773–3780.
28. Margosiak, S. A., D. L. Vanderpool, W. Sisson, C. Pinko, and C. C. Kan. 1996. Dimerization of the human cytomegalovirus protease: kinetic and biochemical characterization of the catalytic homodimer. *Biochemistry* **35**:5300–5307.
29. Martinez, A., A. Castro, C. Gil, and C. Perez. 2001. Recent strategies in the development of new human cytomegalovirus inhibitors. *Med. Res. Rev.* **21**:227–244.
30. Pinto, I. L., R. L. Jarvest, B. Clarke, C. E. Dabrowski, A. Fenwick, M. M. Gorczyca, L. J. Jennings, P. Lavery, E. J. Sternberg, D. G. Tew, and A. West. 1999. Inhibition of human cytomegalovirus protease by enedione derivatives of thieno[2,3-d]oxazinones through a novel dual acylation/alkylation mechanism. *Bioorg. Med. Chem. Lett.* **9**:449–452.
31. Pray, T. R., K. K. Reiling, B. G. Demirjian, and C. S. Craik. 2002. Conformational change coupling the dimerization and activation of KSHV protease. *Biochemistry* **41**:1474–1482.
32. Preston, V. G., J. A. Coates, and F. J. Rixon. 1983. Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. *J. Virol.* **45**:1056–1064.
33. Qiu, X., J. S. Culp, A. G. DiLella, B. Hellmig, S. S. Hoog, C. A. Janson, W. W. Smith, and S. S. Abdel-Meguid. 1996. Unique fold and active site in cytomegalovirus protease. *Nature* **383**:275–279.
34. Robertson, B. J., P. J. McCann III, L. Matusick-Kumar, W. W. Newcomb, J. C. Brown, R. J. Colonno, and M. Gao. 1996. Separate functional domains of the herpes simplex virus type 1 protease: evidence for cleavage inside capsids. *J. Virol.* **70**:4317–4328.
35. Schwede, T., J. Kopp, N. Guex, and M. C. Peitsch. 2003. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* **31**:3381–3385.
36. Shieh, H. S., R. G. Kurumbail, A. M. Stevens, R. A. Stegeman, E. J. Sturman, J. Y. Pak, A. J. Wittwer, M. O. Palmier, R. C. Wiegand, B. C. Holwerda, and W. C. Stallings. 1996. Three-dimensional structure of human cytomegalovirus protease. *Nature* **383**:279–282.
37. Stevens, J. T., C. Mapelli, J. Tsao, M. Hail, D. O'Boyle, II, S. P. Weinheimer, and C. L. Dillani. 1994. In vitro proteolytic activity and active-site identification of the human cytomegalovirus protease. *Eur. J. Biochem.* **226**:361–367.
38. Tong, L., C. Qian, M. J. Massariol, P. R. Bonneau, M. G. Cordingley, and L. Lagace. 1996. A new serine-protease fold revealed by the crystal structure of human cytomegalovirus protease. *Nature* **383**:272–275.
39. Welch, A. R., L. M. McNally, M. R. Hall, and W. Gibson. 1993. Herpesvirus proteinase: site-directed mutagenesis used to study maturational, release, and inactivation cleavage sites of precursor and to identify a possible catalytic site serine and histidine. *J. Virol.* **67**:7360–7372.
40. Wilmanns, M., C. C. Hyde, D. R. Davies, K. Kirschner, and J. N. Jansonius. 1991. Structural conservation in parallel beta/alpha-barrel enzymes that catalyze three sequential reactions in the pathway of tryptophan biosynthesis. *Biochemistry* **30**:9161–9169.
41. Wittwer, A. J., C. L. Funckes-Shippy, and P. J. Hippenmeyer. 2002. Recombinant full-length human cytomegalovirus protease has lower activity than recombinant processed protease domain in purified enzyme and cell-based assays. *Antivir. Res.* **55**:291–306.
42. Yoakim, C., W. W. Ogilvie, D. R. Cameron, C. Chabot, C. Grand-Maitre, I. Guse, B. Hache, S. Kawai, J. Naud, J. A. O'Meara, R. Plante, and R. Deziel. 1998. Potent beta-lactam inhibitors of human cytomegalovirus protease. *Antivir. Chem. Chemother.* **9**:379–387.

3.3. Part III

HTS and hit validation

Abstract

In order to identify HCMV protease inhibitors, the yeast-based Lex-M system (as described in Part I of this thesis) was optimized for high-throughput screening (HTS). An automated screening of a 15'000 small molecule library was performed at 50 μ M final concentration. 67 compounds, which counteracted the growth inhibition caused by HCMV protease in a concentration-dependant way, were selected. These compounds were tested for inhibition of viral propagation in human cells by two different methods, i.e. ELISA and plaque reduction assay (PRA). Though several compounds showed activity in low micromolar IC₅₀ range in both assays, potential effects of compound toxicity on virus proliferation could not be excluded. In order to test whether these selected compounds inhibited HCMV protease activity, we performed *in vitro* FRET assays and analyzed pAP substrate cleavage by electrophoresis and Trp1¹⁹⁴-Me substrate cleavage by Western blotting. However, despite many efforts, we were not able to prove a direct HCMV protease inhibition by our compounds.

1. Material and Methods

1.1. Yeast strains

The VCY3 strain used for screening and the RLY07 strain used for compound testing in the Trp1-M system were described in Material and methods of Part I and Part II, respectively.

1.2. Construction of plasmids

Plasmids expressing the human cytomegalovirus protease (aa 1-256), the LexA-Me-Gal4AD substrate and the Trp1¹⁹⁴-Me substrate were described in Material and methods of Parts I and II.

1.3. The chemical library

The screening library is constituted of 15'000 compounds selected from the commercially available ChemDiv (www.chemdiv.com) library. These 15'000 compounds were chosen by means of a filtering approach called the Lipinski's "rule-of-5" (Lipinski, Lombardo et al. 2001).

1.4. Screening procedure

The yeast strain VCY3 expressing HCMV protease (1-256) from the *GALI* promoter and the LexA-Me-Gal4AD substrate from the truncated *ADHI* promoter was used for screening the 15'000 compound library. The screening was performed in a 96-well format at a final concentration of 50 µM. 80 wells per plate contained 1.5 µL of different test substances dissolved in DMSO at a concentration of 5 mM. As negative control for yeast growth, 8 wells per plate contained 1.5 µL DMSO. The remaining 8 wells contained 1.5 µL of 0.15% glucose as a positive control for yeast growth. Glucose represses expression of HCMV protease driven by the *GALI* promoter and therefore mimics the effect of a HCMV protease antagonist.

The library was divided into 5 batches of 38 plates (batch 5 contained only 36 plates). One batch was screened in 2 days. Yeast preculture was grown overnight in synthetic medium (-Leu/-Trp) containing 2% galactose. The next day, exponentially

growing cultures were washed once with water and diluted to an OD₆₀₀ of 0.001 in 1 L – His/-Leu/-Trp selective medium containing 2% galactose. 150 µL of the diluted cells were added to 96-well plates in a fully automated manner by a Tecan robot. To obtain a blank value (t=0), OD₅₉₅ was measured directly after addition of yeast cells by a microtiter plate reader (GENios Reader, Tecan). The plates were then incubated at 30°C and after 30 s shaking OD₅₉₅ was read after 24, 30 and 36 h.

To evaluate the quality of the screening, the Z' factor (Zhang, Chung et al. 1999) for each screening plate was calculated using the following equation:

$$Z' = 1 - 3 \times (\text{SDEV}_{\text{positive control}} + \text{SDEV}_{\text{negative control}}) / (\text{AV}_{\text{positive control}} - \text{AV}_{\text{negative control}})$$

(SDEV: standard deviation; AV: average)

The Z' factor is the most commonly used metric to define HTS assay quality. An assay with Z'=1 would be an ideal assay. In practice, assays with $1 > Z > 0.5$ are considered as suitable for HTS.

1.5. FRET HCMV protease assay

Recombinant HCMV protease (assemblin) was purchased at BACHEM (H-2138). HCMV protease FRET substrates were also from BACHEM (I-1870 (Ac-tBu-Gly-tBu-Gly-Asn(Me)₂-Ala-7-AMC substrate (Bonneau, Plouffe et al. 1998)) and M-2450 (Abz-tBu-Gly-tBu-Gly-Asn(Me)₂-Ala↓Ser-Ser-Arg-Leu-Tyr(3-NO₂)-Arg-OH substrate (Bonneau, Plouffe et al. 1998)).

To perform the biochemical assay, the protease was diluted to a 2.5 µM stock solution in assay buffer (50 mM Tris/HCl pH8, 0.5 M Na₂SO₄, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 0.05% casein), and the substrates to a 1 mM stock solution in DMSO. Inhibitors were dissolved in DMSO as 20x solution. Assays were performed in 25 µL reactions with 50 nM protease and 5 µM substrate. The enzymatic reactions were carried out in black, opaque polystyrene 384 well plates (Corning, # 3676). 1.25 µL of 20x compound solution was distributed per well and mixed with 11,25 µL of 100 nM protease dissolved in assay buffer. The plate was then incubated 30 min at 30°C, and 12.5 µL of 10 µM substrate solution was added. Progress curves were monitored at Ex 360/Em 440 nm for the AMC substrate and Ex 312/Em 415 nm for the Abz substrate on a Tecan GENios Reader. Compounds were tested in triplicate, at concentrations where no precipitation, autofluorescence and quenching could be observed.

1.6. Cells

Human embryonic lung fibroblasts (MRC5) and human embryonic kidney epithelial cells (HEK 293) were obtained from the American Type Culture Collection (CRL-1573 and CCL-171). MRC5 cells were grown in minimum essential medium Eagle (MEM, Gibco) supplemented with 2 mM L-glutamine and Earle's BSS (1.5 g/L sodium bicarbonate), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 % fetal calf serum (FCS), and used between culture passages 18 and 21. HEK 293 were grown in Dulbecco's modified Eagle Medium (DMEM, Gibco) supplemented with 5% FCS. Cell cultures were incubated at 37°C in a 5% CO₂ atmosphere.

1.7. Viruses

HCMV (strain AD169, ATCC number VR-538) stocks were prepared by propagating the virus in a MRC5 cell culture. When 90-100% of the cells showed symptoms of infection, supernatant was removed, cells were trypsinized and collected in complete MEM + 5% FCS + 5% DMSO. This cell-associated inoculum was aliquoted and stored at -80°C.

Coxsackievirus B3 (Strain Nancy, ATCC number VR-30) stocks were prepared by propagating the virus in a HEK 293 cell culture. When 90-100% of the cells were floating in the medium, the medium containing the non-adsorbing cells was collected and centrifuged 3 min at 800 rpm. Supernatant was removed and the cells were resuspended in a small volume of complete MEM + 2% FCS (about 10% of the previous volume) and transferred to Eppendorf tubes. After 3 freeze-thaw cycles, Eppendorf tubes were centrifuged for 5 min at 10'000 rpm at 4°C, and the supernatant containing free viruses was collected. 5% DMSO were added to the virus suspension, which was subsequently aliquoted and stored at -80°C. Infectivity of both virus stocks was determined by end point titration (Spearman and Kärber, 1912).

1.8. Antiviral ELISA assay

1. Infection with HCMV:

10^4 MRC5 cells were distributed per well in 96-well plates (NUNC, # 167008) in a volume of 100 μ L of assay medium (complete MEM as defined above+ 5% FCS). For each compound concentration, 3 wells were infected with 100 PFU of HCMV diluted in 50 μ L assay medium, and 1 control well received only 50 μ L assay medium. Plates were then incubated at 37°C for 48 h. Compounds were diluted in assay medium at 4x the final concentration, and 50 μ L were added per well. Ganciclovir at 4 μ M (final concentration) was used as positive control, negative control cells were treated with DMSO. The plates were then incubated for further 5 days.

2. Infection with CVB3:

3×10^4 HEK 293 cells were seeded per well in 96-well plates in a volume of 100 μ L of assay medium (DMEM + 2% FCS) and infected with 100 PFU of CVB3 resuspended in a volume of 50 μ L assay medium. Compounds were added simultaneously to the virus suspension as described for HCMV and the plates were incubated for 24 h at 37°C.

3. ELISA

Medium was tipped from the plates and the cell monolayers were washed 1x by gentle immersion in phosphate buffered saline (PBS). PBS was then removed and the cells were fixed by addition of 100 μ L of 1:1 mix of acetone and methanol for 3 min. Following a further wash with PBS, plates were blocked with 100 μ L of PBS containing 0.05% Tween 20 and 2% skimmed milk powder at 37°C for 30 min. The plates were then washed 1x with PBS + 0.05% Tween 20, and 50 μ L of anti-CMV antibody (mouse monoclonal antibody directed against gB late cytoplasmic antigen, ANAWA) diluted 1:500, or anti-CV antibody (mouse monoclonal antibody, Chemicon International) diluted 1:1000, was added to each well. After 2 h incubation at 37°C, plates were washed 3x with PBS/Tween, and 50 μ L of goat anti-mouse IgG antibody conjugated to horseradish peroxidase

(PIERCE) diluted 1:1'000 was added to the wells. The plates were incubated for another hour, washed thoroughly 5x and dried. 50 μ L substrate solution (BM blue POD, Roche) was added per well, and color allowed to develop at room temperature. The reaction was stopped by the addition of 50 μ L 1 M HCl, and the plates were read spectrophotometrically at a wavelength of 450 nm.

The mean color development of triplicate infected wells at each compound concentration was calculated as a percentage of the mean adsorption of untreated, infected controls after both values had been adjusted for nonspecific background. These percentage inhibition values were plotted against compound concentration and the 50% inhibitory concentration (IC_{50}) derived by regression analysis (graph prism).

1.9. XTT assay

The XTT assay was performed in parallel to the ELISA assay. Cell seeding and compound treatment were done as described for the ELISA assay, except that phenolred-free media were used and that no virus suspension was added to the cells. An XTT (AppliChem, # 111072-31-2) working solution was prepared by adding 25 μ M of PMS to 1 mg/mL of XTT diluted in phenolred-free medium. 50 μ L of this working solution was added to each well and OD_{450} was measured after 1 h.

1.10. Plaque reduction assay (PRA)

10^6 MRC5 cells were seeded in 5 cm diameter dishes with grid, in 4 mL of complete MEM + 10% FCS. 3 rectangles of 4 cm x 1 cm had been drawn on each dish to facilitate the counting of the plaques afterwards. After 24 h, confluent monolayers were infected during 2 h with 2 mL of HCMV (strain AD169) inoculum at an infectivity ratio of 400 PFU/mL. Thereafter, unadsorbed viruses were removed from the plates and compounds diluted in complete MEM + 5%FCS were added. After 7 days of incubation at 37°C, cells were washed once with PBS, fixed with 10% formaldehyde and stained with 0.8% crystal violet (Sigma) prepared in 20% ethanol. The viral plaques were counted microscopically and IC_{50} values were determined by linear regression analysis.

1.11. HCMV protease *in vitro* tests performed in Wade Gibson's laboratory

1. Preparation of enzyme.

Assemblin containing a mutation at the I site and an N-terminal 6x His-tag was expressed in *E. coli*. A lysate was prepared in 8 M urea and assemblin was purified on a Ni-NTA column into 2 M urea. Assemblin was exchanged into refolding buffer (100 mM Na₂PO₄, pH8, 300 mM NaCl, 50 mM DTT, 15% glycerol). The concentration of enzyme was estimated on a SYPRO-Ruby stained SDS-PAGE by comparing with BSA standards run on the same gel.

2. Preparation of substrate.

SCMV pAP containing both a 6xHis-tag and a HWHWH-tag at its N-terminus was prepared and purified into 2 M urea as above. pAP concentration was estimated by measuring absorbance at 280 nm to be approximately 6 μM.

3. Inhibition reactions.

A 10-fold dilution series (3 mM to 3 μM) of each compound was prepared in DMSO. 10 μL of each inhibitor was added to 40 μL ddH₂O and 50 μL 2x reaction buffer (100 mM Tris pH8, 1 M Na₂SO₄, 0.2 mM EDTA, 2 mM TCEP). 275 μL purified substrate was added to 275 μL 2x reaction buffer. 55 μL refolded assemblin (~3 μM) was added to 220 μL refolding buffer and 275 μL 2x reaction buffer. 10 μL substrate mix was added to 50 microfuge tubes, then 10 μL inhibitor was added to each tube, vortexed and briefly centrifuged. In 20 s intervals, 10 μL of enzyme mix was added, mixed by pipetting 3x and vortexing quickly on a low setting. After a 1 h incubation at room temperature 10 μL of 4x SDS-PAGE sample buffer was added in 20 s intervals and mixed thoroughly by pipetting and vortexing. Samples were stored at -80 C until analysis by SDS-PAGE.

2. Results

2.1. Optimization of the LexA-M yeast system for automated screening

In order to identify small molecule inhibitors of HCMV protease, the yeast growth selection system based on reporter gene activation as described in Part I was applied to a high-throughput screening (HTS). A first batch of 3'040 compounds from the ChemDiv library was distributed in 38 96-well plates containing each 80 test compounds and was then screened against a VCY3 strain expressing the HCMV protease and its LexA-Me-Gal4AD substrate. As specified in Part I, expression of HCMV protease was controlled by the *ACT4* promoter and LexA-Me-Gal4AD by a truncated *ADHI* promoter. Compounds were screened at a final concentration of ~50 μ M for their ability to restore cell proliferation. A detailed description of the screening procedure is provided in Material and methods.

Upon analysis of the preliminary results, we noticed that a problem occurred during this first screening phase: in each microtiter plate, OD₆₀₀ in the wells of columns 1, 4, 8 and 11 was systematically higher than in the other wells (Figure 1). OD₆₀₀ in columns 2, 5, and 9 was in the opposite lower, and this trend was independent of the nature of the compounds present in these particular columns. More precisely, the average of OD₆₀₀ in column 8 (column exhibiting the highest OD₆₀₀) after 30 hours incubation at 30°C was 35% higher than the OD₆₀₀ in column 2 (column exhibiting the lowest OD₆₀₀). Given that hits are usually selected when they stimulate proliferation of the target expressing cells more than 5% or 10% relative to proliferation of the negative control (the same cells grown in the presence of DMSO), it is clear that the system as such was not usable for screening.

To find solutions to this problem, we performed control tests where yeasts were grown in 96-well plates containing 1.5 μ L DMSO per well instead of compound solutions. First, we removed glycerol from the assay medium, which reduced the “wavy pattern” to an OD₆₀₀ difference of about 10% between column 2 and 8. In a second step 3-AT was removed and the difference was further reduced to 5%. It seems that this “wavy pattern” was related to the robot mode of pipetting (robot fills 4 columns per aspiration step, what correlates with OD₆₀₀ being always higher in the 4th column), however the cause of the phenomenon remains unexplained.

Thus, the LexA-M system as described in Part I was modified so that it could be used without glycerol and 3-AT. This reduced dramatically the difference of proliferation rates between yeasts expressing the substrate alone and yeasts expressing the substrate and its protease. To extend the window between these two strain proliferation rates, we expressed the protease from the strongest promoter *GALI* instead of the *ACT4* promoter, and the inoculation OD₆₀₀, previously defined at 0.01, was decreased to 0.001. Despite these modifications, the 4-fold difference of proliferation rates measured in presence of glycerol and 3-AT, was reduced to 1.6 fold with the new assay conformation. The Z' factor was lowered from 0.62 to 0.54. Table 1 summarizes the modifications that were applied to the screening system and the consequences of these modifications.

2.2. Screening and hit confirmation in yeast

The 15'000 compound library was screened in this modified LexA-M system. An overview of the experimental procedure for the screening and for further characterization of the primary hits is outlined in Figure 2. A primary hit was defined as a compound that stimulates growth of HCMV protease expressing cultures more than 8% relative to growth of the negative control. Using this hit criterion, 259 compounds were found to be active in our assay. Among the 259 primary hits, 84 were confirmed to stimulate growth of HCMV protease expressing cultures more than 5% at any or all of these three concentrations: 50 µM, 25 µM and 12.5 µM.

The fact that many screening hits are nonspecific represents a serious problem in early drug discovery. As mentioned in Part I, cell reporter system are prone to select for false positives interfering with LexA dimerization, LexA binding to DNA, with the transcription, translation or activity of His3p reporter protein, with the expression of LexA-M substrate or HCMV protease. It is expected however that *bona fide* inhibitors stimulate the proliferation of cells in a target-dependant manner. In order to eliminate unspecific compounds, the 84 hits were tested at 50 µM, 25 µM and 12.5 µM against the strain expressing the LexA-Me-Gal4AD substrate alone. 17 compounds stimulated growth in this strain at least at one concentration and were therefore discarded. The other 67 compounds were considered as confirmed hits.

2.3. *In vitro* FRET analysis of putative HCMV protease inhibitors

In order to clarify whether the 67 hits identified in the screening directly inhibit the protease, they were tested in a fluorescence resonance energy transfer (FRET)-based *in vitro* assay, using the amino-4-methylcoumarin (AMC) substrate that is described in the Material and methods section. BI36 compound was used as positive control for this assay, and the calculated IC₅₀ of 0.3 µM obtained in house was the same as the published IC₅₀ (0.5 µM) (Yoakim, Ogilvie et al. 1998). 41 test compounds were inactive up to 100 µM concentration and the remaining 26 compounds could not be evaluated due to precipitation, autofluorescence or quenching. Thus, none of the 67 test compounds was scored as positive in this biochemical assay. In order to circumvent the problems of fluorescence and quenching, another FRET substrate (Abz) was used, which absorbed and emitted at different wavelengths as AMC substrate. Yet, these problems were present at different degree at both wavelengths assessed.

Even though the results of the biochemical assays did not show direct targeting of the protease, 44 compounds were reordered on the basis of their ability to promote proliferation of HCMV protease expressing yeast cells and of their protease specificity (they did not stimulate growth of the cells expressing the substrate alone, and they were also not selected in other in-house yeast screenings against other targets: BACE, P2A, c-Met, EphB4).

2.4. Antiviral assay for the evaluation of putative HCMV protease inhibitors

In order to evaluate the potential of compounds to reduce cytomegalovirus proliferation in cell culture, we performed antiviral assays in mammalian cells. For this we used two different assays: an ELISA (Stahle, Schloss et al. 1998; Borthwick, Davies et al. 2003) to make a first selection of compounds potentially active against HCMV, and then a plaque reduction assay (PRA) (Landry, Stanat et al. 2000) to confirm compounds that were positive in the ELISA.

Both GCV and BI36 positive controls were tested in ELISA, and the IC₅₀ calculated for these drugs was very close to the IC₅₀ published in the literature. IC₅₀ values between 0.29 µM and 11 µM were determined for GCV in PRA assays (Landry, Stanat et al. 2000), and our in house ELISA assay delivered for GCV an IC₅₀ of 0.6 µM.

Similarly, we obtained a 60 μM IC_{50} for BI36, which is very close from the 78 μM IC_{50} published by Yoakim et al. (Yoakim, Ogilvie et al. 1998).

In contrast to the *in vitro* FRET assay, the evaluation of hits in the ELISA assay delivered several positives. Out of the 44 compounds tested, 11 showed activity against HCMV. Experiments with concentration ranges from 3 μM to 100 μM enabled to determine compound IC_{50} values with potencies varying from 8 μM for 150E10 to 65 μM for 71E2. A detail of each compound potency is given in Table II. In parallel to the ELISA assay, compound toxicity was assessed in an XTT cell viability assay (Roehm, Rodgers et al. 1991) (Table II). It has to be noticed that for many compounds, such as 150E10, 107F6 and 107H6, a higher toxicity correlates with a better inhibitory potency. It is not possible however to determine if this effect is just a coincidence or if the intrinsic toxicity of the compounds has an effect on virus propagation. To calculate the viral 50% inhibition concentration (IC_{50}), only compound concentrations that permitted at least 80% cell viability in comparison to the DMSO control were taken into account.

In order to confirm these results, we tested the best candidates in a plaque reduction assay (PRA), which is the gold standard for antiviral susceptibility testing. Out of the 9 compounds tested in PRA assay, 8 obtained IC_{50} values in the same range as those calculated in ELISA (Table II).

2.5. Evaluation of compound specificity towards HCMV by coxsackievirus (CV) inhibition tests

8 compounds were confirmed as cytomegalovirus inhibitors in ELISA and PRA assays. In order to establish if these compounds were specific for HCMV or if they would also inhibit other viruses, we infected HEK293 cells with CV and measured via an ELISA assay if our compounds were effective in preventing CV proliferation. Like HCMV, CV relies on a protease for its replication, but the two proteases have different modes of action: the HCMV protease uses a serine to catalyze the hydrolysis reaction, whereas the CV protease works with a cysteine. Thus, we anticipated that our putative HCMV protease inhibitors would not inhibit CV. However, on the 12 compounds tested (Table III), only 4 were specific for HCMV and did not inhibit CV. These compounds included ganciclovir, 19A2, 38F7 and 107H6. The 8 other compounds lowered the titer of CV in culture, and among them BI31 control was very potent (with an IC_{50} of 13.2 μM) against

this virus. Compound 150E10 was again the most efficient inhibitor of the batch with an IC_{50} of 8.4 μ M, i.e. the same IC_{50} as the one calculated for HCMV. And again, like in the HCMV ELISA assay, the compounds that exhibited the highest toxicity were also the most potent against CV. To investigate whether compound toxicity could *per se* inhibit virus propagation, we tested if unrelated compounds that had been selected in screens targeting EphB4 kinase and β -secretase and that were moderately toxic also had an effect on virus proliferation. The 3 compounds presented on Figure 3, which toxicity varied from 15% to 50% at 100 μ M, were shown to reduce HCMV proliferation in ELISA assay. It was verified that these randomly chosen compounds had no effect on HCMV protease in FRET and yeast assays.

To summarize these antiviral assays, 8 test compounds out of 44 were potent against HCMV in both ELISA and PRA assay. However, 5 of them also inhibited CV, and we demonstrated that unrelated toxic compounds could also lower virus titer in ELISA assay. It has to be underlined however that out of the 44 compounds tested in ELISA against HCMV, 9 were toxic and showed no efficiency against the virus.

2.6. Back to yeasts: how do hits perform in assays with glycerol?

As described in paragraph III.2.1, the primary screening in yeast had to be performed without glycerol and 3-AT, despite that glycerol was a key element for the catalytic efficiency of HCMV protease. Screening without glycerol was necessary to prevent the high cell density variations that were produced by the robot in 96-well plates. It was then decided to keep the same conditions to confirm the positives after the primary screening. But at the current stage of the compound characterization, we were interested to know how these compounds would perform in the different yeast assays under optimal conditions: in the LexA-M system with 10% glycerol and 10 mM 3-AT as described in Part I, and in the Trp1-M system with 10% glycerol, as described in Part II.

Therefore, 10 hits positive in ELISA were tested at 50 μ M, 16.6 μ M and 5.5 μ M in these two systems. The results of these growth assays for the 36 hours time point and the 50 μ M concentration are summarized in tables IV and V. The BI31 and BI36 controls were the strongest growth activators in both assays, and this despite their toxicity. Generally, Trp1-M system delivered more positives than LexA-M system, and the amplitude of the stimulation was bigger. Only 107E7 and 150E10 were scored as

positives in both LexA-M and Trp1-M systems. However, it has to be underlined that the data presented in the tables III and IV represent only one time point and one compound concentration. Several compounds were scored as positives at other time points or concentrations. As a matter of fact, most compounds activated cell proliferation in certain conditions, but not in others. Only BI compounds activated growth at every time points and concentrations.

2.7. Test of published HCMV protease inhibitors in the automated screening set-up

At the time when the HTS was done no HCMV protease inhibitor was available. The BI31, BI35 and BI36 compounds (Yoakim, Ogilvie et al. 1998) were sent to us by Boehringer Ingelheim only several months later. Instead of a protease antagonist, 0.15% glucose was used as positive control, because glucose represses the expression of HCMV protease driven by the *GALI* promoter and so mimics the effect of a HCMV protease inhibitor. Control experiments were performed with the series of truncated *GALI* promoters to ensure that partial reduction of HCMV protease activity was detectable. However, considering the suboptimal conditions that were used during this HTS (III.2.1) and the low rate of hit confirmation in yeast LexA-M and Trp1-M systems with glycerol (III.2.6), it was important to find out whether all three BI compounds would have been scored as positives under the automated screening conditions.

Therefore, cells were transformed with the LexA-Me-Gal4AD substrate and the HCMV protease expressed from the *GALI* promoter and were inoculated with a start OD₆₀₀ of 0.001 in selective medium containing 2% galactose and the 3 BI compounds at 50 µM, 16.6 µM and 5.5 µM. Pipetting was done by hand. This experiment showed that only BI36 would have been scored as positive in the 50 µM primary screening. With 15% growth activation at 50 µM after 36 hours of incubation, this compound boosted cell density over the 8% stimulation threshold (Table VI). Inhibition of HCMV protease by BI31 and BI35 compounds at 50 µM did not stimulate growth sufficiently to compensate for their pronounced toxicity. BI35 stimulated cell proliferation above the 8% threshold at 16.6 µM and 5.5 µM at all three time points, but BI31 never passed this limit. In the introduction of this thesis I emphasize that the utilization of cellular assays for screening enables the selection of non-toxic compounds. The non-selection of BI31 and BI35 by the automated LexA-M system could then be expected considering their toxicity. However,

given that these compounds activate strongly in both LexA-M and Trp1-M systems in presence of glycerol, these negative results are not in favor of the LexA-M system as it was used for automated screening. Best example is given by BI36, which at 50 μ M had passed the IC₅₀ in the systems with glycerol (II.3.5). In the automated screening configuration, it stimulated cell proliferation only 15% at 50 μ M, when a complete protease inactivation would produce 60% stimulation. Thus, this experiment shows that the LexA-M system as it was used in automated screening was not optimal and that an alternative would have to be searched to solve the “wavy pattern” that resulted from robot pipetting.

2.8. Western blot to show direct effect of compounds on protease activity

8 compounds had shown antiviral properties in both ELISA and PRA assays, and some of them such as for example 150E10 or 107H6 even presented IC₅₀ values in the low micromolar range. However, some concerns were raised regarding a potential effect of their toxicity on the virus proliferation, and we needed to demonstrate that these compounds directly targeted protease activity. So far, none of these 8 compounds was shown to be active in *in vitro* assays, and their efficiency in yeast and in mammalian cell assays could be attributed to a mechanism independent of HCMV protease inhibition.

In order to find out whether the compounds directly inhibited the protease, Western blot analysis was performed as exemplified with BI36 in the Part II of this thesis. RLY07 strain expressing Trp1¹⁹⁴-Me substrate and HCMV protease was incubated during 16 hours in growth medium containing the test compounds, BI36 (positive control) or DMSO (negative control). Extracts were loaded on an SDS-gel and analyzed by Western blot. Figure 4 shows that BI36 control as well as 19A3 (weakly), 71E2, 112H10 and 150E10 seem to prevent cleavage of Trp1¹⁹⁴-Me substrate. However, similarly to the results presented in paragraphs II.3.3 and II.3.5, no cleavage product could be detected after proteolysis. It was therefore impossible to know if test compounds really inhibited cleavage or if they caused the overexpression of the Trp1¹⁹⁴-Me substrate. In the same manner, it was impossible to discriminate whether compound caused the underexpression of the protease or if they inhibited it.

Therefore, many efforts were invested to detect those proteolysis fragments, which should migrate at 24 kDa (N-terminus) and 6 kDa proteins (C-terminus). 1) In

order to improve the signal given by the Trp1¹⁹⁴-Me substrate and its cleavage products, different tags were tested. FLAG, Myc, and triple-HA were added to the N-terminus, but cleavage fragments remained undetected. 2) Presuming that the C-terminal 6 kDa fragment would be too small to be detected we fused a 26 kDa GST group at the end of the Trp1¹⁹⁴-Me molecule. But neither the 24 kDa N-terminal fragment nor the new 30 kDa C-terminal fragment could be detected. 3) To make sure that fragments were not below the detection threshold of the Western blot Trp1¹⁹⁴-Me substrate was expressed from the *ACT4* promoter, which is stronger than the truncated *ADHI* promoter that was used so far. But still at this high expression level no 24 kDa band was detected. 4) MG132 proteasome inhibitor was added to the growth medium to prevent post-cleavage degradation of Trp1¹⁹⁴-Me fragments. This alternative approach also did not allow detection of proteolysis fragments.

Thus, despite many efforts, we were not able to detect a product resulting from the cleavage of Trp1¹⁹⁴-Me substrate by the HCMV protease. As a consequence, we cannot unequivocally conclude that compounds inhibit protease activity.

2.9. Effect of hits on *in vitro* cleavage of pAP

Cleavage inhibition of different FRET substrates has been investigated in biochemical assays (III.2.3). But several hits were either fluorescent or quenchers and could therefore not be tested. All commercially available substrates being small peptides (6-12 amino acids), their cleavage cannot be addressed by electrophoresis. I found no way to demonstrate in house a direct effect of our compounds on proteolysis by HCMV protease. Therefore, I requested the collaboration of Professor Wade Gibson (Johns Hopkins University School of Medicine, Baltimore), who has a long time experience with HCMV protease. He performed the following experiment: the natural HCMV protease substrate, pAP, was incubated during 1 hour with assemblin and compounds at 0.1 μ M, 1 μ M, 10 μ M and 100 μ M. Then reactions were loaded on an electrophoresis gel, and proteins were colored with SYPRO-Ruby stain. Results are depicted on Figure 5. The 41 kDa full-length pAP (lane 1) was cleaved at the M-site and fragments of 35 kDa and 6 kDa (the smaller is not visible on the gel) were released (lane 2). On the right, BI36 control clearly inhibits proteolysis of pAP at 100 μ M and 10 μ M. By contrast, none of the hits inhibits

HCMV protease in this assay. Thus, it seems that none of our hits is active *in vitro* on the HCMV protease.

3. Discussion

In order to select for HCMV protease inhibitors, the LexA-M system that was developed in Part I of this thesis had to be modified to suit to automated screening conditions. A 15'000 small molecule library was screened and 67 hits were selected for their ability to stimulate proliferation of the protease-expressing strain above an 8% threshold. These compounds activated growth in a protease-dependant and dose-dependant way.

These hits were further tested in mammalian cell assays (ELISA and PRA), and, among them, 8 compounds showed good antiviral properties. It was not clear though, if this effect was due to the inhibition of the viral protease or to general toxicity of the compounds. In attempts to show that these compounds were targeting the protease, we tested them first in *in vitro* FRET assays, where they were either inactive or could not be investigated because of their fluorescence or quenching properties. In an additional test, their influence on the cleavage of the TRP1¹⁹⁴-M substrate in yeast was evaluated by Western blot. However, this experiment did not allow discriminating between *bona fide* inhibitors and compounds that would modify substrate or protease expression. Finally, the ability of compounds to prevent the cleavage of the natural substrate pAP *in vitro* was examined. In this assay performed in the laboratory of Professor Gibson (Johns Hopkins University School of Medicine, Baltimore) none of the hits was able to prevent cleavage under these experimental conditions. Thus, no evidence of direct inhibition of HCMV protease by the identified hits could be produced by all these experiments taken together.

3.1. Reasons for the lack of direct protease inhibition of the selected hit compounds

There are several explanations why no inhibitor has been identified, which would have been active not only in yeast and mammalian cell assays, but also in biochemical assays. First, as discussed in chapter III.2.1 and III.2.7, the conditions applied for HTS were not optimal. In the absence of glycerol, the sensitivity of the LexA-M system was reduced. This was illustrated by the BI36 control. This compound, a potent HCMV protease inhibitor, stimulated growth only 15% as compared to 94% in the same system with glycerol. If another HTS had to be performed, we would add 10% glycerol to the selection medium and solve the “wavy pattern” problem (as described in III.2.1) such that

each screening plate column would be filled separately. In such a setup, each aspiration/distribution step would lead to the filling of one column, as compared to the filling of 4 columns per aspiration step in the original screening.

Another argument to explain the lack of convincing inhibitors is the limited size of the library. Indeed, our library consists of only 15'000 compounds, which is far away from the typical multimillion compound libraries of pharmaceutical companies (Coburn, Stachel et al. 2004).

Finally, it has to be underlined that HCMV protease is a difficult target for all types of screening assays. This enzyme was first described in 1991 (Welch, Woods et al. 1991), and crystallized in 1996 (Chen, Tsuge et al. 1996; Qiu, Culp et al. 1996; Shieh, Kurumbail et al. 1996; Tong, Qian et al. 1996). Intensive researches were carried out to identify new protease-targeting drugs that would replace or complement HCMV current therapies. However, no inhibitor entered clinical trials so far. The difficulty with HCMV protease resides in its poor activity (k_{cat} 20min⁻¹; (Pinko, Margosiak et al. 1995)) and in its status of obligate dimer (K_d 8μM; (Margosiak, Vanderpool et al. 1996)). Moreover, in opposition to other enzymes where the active site is nested in a deep cleft, the active site of HCMV protease is shallow (Chen, Tsuge et al. 1996). As a result, chemical compounds have few possibilities to bind. Therefore, most inhibitors published so far bind covalently to the enzyme, either by alkylation of the active Ser 132 (Deziel and Malenfant 1998; Borthwick, Exall et al. 2002), or by oxidation of cysteine residues (Baum, Ding et al. 1996). Since potential interactions between the enzyme and its inhibitors are reduced due to shallowness of the active site, it is also difficult to design inhibitors that are selective. The possibility of cross-reactivity with other enzymes, particularly proteases, becomes a concern.

3.2. Dimerization inhibitors versus active site inhibitors

So far, the strategies to target the herpesviral proteases were mostly based on active site inhibition. However, allosteric inhibition is gaining interest. It was shown for example that dimer disruption totally inhibits KSHV protease activity (Shimba, Nomura et al. 2004). In the same way, single mutations at the dimer interface of HCMV protease, even if they do not impair dimerization, inhibit catalytic efficiency (Batra 2001). A mutagenesis study of KSHV protease (Pray, Reiling et al. 2002) showed that the dimer

interface is finely developed and sensitive to perturbations, which in turn greatly affect the activity of the enzyme. Thus, blocking the dimerization or altering the dimer interface would be as efficient as blocking the active site. Since the herpesviral protease interface consists of unique surface area, with deep grooves and large hydrophobic pockets (Chen, Tsuge et al. 1996), there are theoretically many binding possibilities for potent and specific inhibitory molecules.

However, such dimerization inhibitors might not be selected by *in vitro* screening campaigns as they are usually performed. Indeed, to be able to screen with a reasonably low concentration of enzyme, researchers usually work with high concentrations of salt in the assay buffer, which artificially lower the K_d . Thus, it is possible to have an HCMV protease active at 50 nM, whereas natural K_d of the enzyme is 8 μ M (Margosiak, Vanderpool et al. 1996). But we do not know if a dimerization inhibitor with a K_i of for example 300 nM would be able to compete with another monomer in such conditions. To evaluate the potency of a compound that disrupts dimerization, the assay should be performed in buffer containing physiological salt concentrations.

These physiological conditions are precisely present in our yeast assays. Compounds are tested in a cytoplasmic environment, where no salt has been added. Thus, we might have selected dimerization inhibitors, and this could explain why none of our hits showed activity *in vitro*. There are other cases of compounds that are poorly active in biochemical assays but very potent in cell culture, such as, for example, Glivec, the first tyrosine-kinase inhibitor to be approved for the treatment of cancer. This small molecule binds to the inactive form of the oncogenic kinase BCR-ABL (Schindler, Bornmann et al. 2000) and prevents its autophosphorylation. Since biochemical assays are usually performed with the active form of the enzyme, Glivec was not potent *in vitro*.

In order to test whether selected hits disrupt dimerization of HCMV protease, a size exclusion chromatography could be performed. At 8 μ M concentration the protease is in equilibrium between monomeric and dimeric enzyme configurations (Margosiak, Vanderpool et al. 1996). Chromatography should elucidate if the presence of a compound shifts this equilibrium towards monomeric state or not. However, this experiment would necessitate infrastructure and resources that we do not have.

3.3. Compounds active in antiviral assays: protease inhibitors or false positives?

A big concern after this screening was the reliability of the inhibitors selected. No proof could be made that they directly targeted HCMV protease activity, neither in biochemical assays nor in Western blots. Moreover, even if they obviously inhibited virus proliferation in human cell assays, it was not clear if this effect was really due to protease inhibition or if it was an artifact caused by their toxicity. Indeed, since viruses use the cell machinery to replicate it is conceivable that altered cellular metabolism retard virus proliferation. An unsought experiment gave some credit to this hypothesis: during a weekend the temperature of the 37°C incubator dropped to 31°C when the virus culture was between the 4th and the 7th day of infection. As a consequence, no cytopathic effect (CPE) could be observed in these cultures after 7 days, whereas under normal conditions (37°C), large plaques are visible (data not shown). This shows that suboptimal cellular conditions impair virus proliferation.

In paragraph III.2.5, the specificity of compounds towards HCMV was tested by examining their effect on coxsackievirus (CV). It was shown that several hits inhibited CV and were therefore not specific. However, it is important to mention that the essential protease of *picornaviridae*, the picornain cysteine 3C protease, shares similarities with HCMV protease. Crystal structures of different picornaviral 3C proteases resemble to those of the chymotrypsin family of serine proteases with the active site serine being replaced by a cysteine residue (Allaire, Chernaia et al. 1994; Malcolm 1995). The replacement of active site cysteine of poliovirus and CV 3C proteases with a serine results in catalytically active enzymes (Lawson and Semler 1990). Like herpesviruses proteases, 3C proteases are slow acting enzymes (Malcolm 1995), their active site is also located in a shallow cleft (Allaire, Chernaia et al. 1994), and the third member of their catalytic triad has only a minor role in catalytic mechanism (Allaire, Chernaia et al. 1994). Importantly, these proteases are inhibited by related active site inhibitors, respectively β -lactams for herpesviruses proteases (Yoakim, Ogilvie et al. 1998; Gerona-Navarro, Perez de Vega et al. 2004) and β -lactones for picornaviruses proteases (Lall, Ramtohul et al. 2002). It is thus conceivable that inhibitors could inhibit both proteases, as exemplified by the β -lactam BI31 in Part III.5. However, if our putative inhibitors would inhibit both protease active sites, they should be active in biochemical assays and they were not. And if they would prevent dimerization they should be specific for

HCMV, since picornaviruses proteases do not need to dimerize to be active (Peters, Kusov et al. 2005). 3 out of the 10 hits of table VII were selective towards HCMV; they were yet inactive in both yeast assays with glycerol. The other hits were not selective and inactive in biochemical assay.

3.4. Chemical structure comparison between hits and validated HCMV protease inhibitors

To investigate whether our putative inhibitors shared common chemical structures with known HCMV protease antagonists we compared the scaffold of published inhibitors with the structure of our hits. Interestingly, several hits are built on the same scaffold like known inhibitors. For example, compounds 107E7, 107F6 and 107H6 are isocoumarin derivatives. And 3,4-dichloroisocoumarin is a general serine inhibitor, which was shown to inhibit HCMV protease *in vitro* with an IC_{50} of 4 μ M (Levine, Michener et al. 1997) (Figure 6A). Similarly, compound 54C8, a benzoxazinone derivative, was selected in the yeast screening but showed no activity in antiviral assays. This compound is built on the same scaffold like several herpesviral protease inhibitors that show IC_{50} values in the low micromolar range (Jarvest, Parratt et al. 1996; Abood, Schretzman et al. 1997) (Figure 6B). Unfortunately, no compound in the library was built on lactam scaffolds. We can then not determine if they would have been selected. However, we showed that the β -lactam BI36 would have been scored as positive in the yeast screening, and that it activated strongly in both LexA-M and Trp1-M assays with glycerol.

4. References

- Abood, N. A., L. A. Schretzman, et al. (1997). "Inhibition of human cytomegalovirus protease by benzoxazinones and evidence of antiviral activity in cell culture." Bioorg Med Chem Lett **7**(16): 2105-2108.
- Allaire, M., M. M. Chernaia, et al. (1994). "Picornaviral 3C cysteine proteinases have a fold similar to chymotrypsin-like serine proteinases." Nature **369**(6475): 72-6.
- Batra, R. (2001). "Molecular mechanism for dimerization to regulate the catalytic activity of human cytomegalovirus protease. [see comments.]" Nat Struct Biol **8**(9)(Sept.): 810-7.
- Baum, E. Z., W. D. Ding, et al. (1996). "Flavins inhibit human cytomegalovirus UL80 protease via disulfide bond formation." Biochemistry **35**(18): 5847-55.
- Bonneau, P. R., C. Plouffe, et al. (1998). "Design of fluorogenic peptide substrates for human cytomegalovirus protease based on structure-activity relationship studies." Anal Biochem **255**(1): 59-65.
- Borthwick, A. D., D. E. Davies, et al. (2003). "Design and synthesis of pyrrolidine-5,5'-trans-lactams (5-oxo-hexahydropyrrolo[3,2-b]pyrroles) as novel mechanism-based inhibitors of human cytomegalovirus protease. 4. Antiviral activity and plasma stability." J Med Chem **46**(21): 4428-49.
- Borthwick, A. D., A. M. Exall, et al. (2002). "Pyrrolidine-5,5-trans-lactams as novel mechanism-based inhibitors of human cytomegalovirus protease. Part 3: potency and plasma stability." Bioorg Med Chem Lett **12**(13): 1719-22.
- Chen, P., H. Tsuge, et al. (1996). "Structure of the human cytomegalovirus protease catalytic domain reveals a novel serine protease fold and catalytic triad." Cell **86**(5): 835-43.
- Coburn, C. A., S. J. Stachel, et al. (2004). "Identification of a small molecule nonpeptide active site beta-secretase inhibitor that displays a nontraditional binding mode for aspartyl proteases." J Med Chem **47**(25): 6117-9.
- Deziel, R. and E. Malenfant (1998). "Inhibition of human cytomegalovirus protease N(o) with monocyclic beta-lactams." Bioorg Med Chem Lett **8**(11): 1437-42.

- Gerona-Navarro, G., M. J. Perez de Vega, et al. (2004). "Synthesis and anti-HCMV activity of 1-acyl-beta-lactams and 1-acylazetidines derived from phenylalanine." Bioorg Med Chem Lett **14**(9): 2253-6.
- Jarvest, R., M. Parratt, et al. (1996). "Inhibition of HSV-1 protease by benzoxazinones." Bioorg Med Chem Lett **6**(20): 2463-2466.
- Lall, M. S., Y. K. Ramtohl, et al. (2002). "Serine and threonine beta-lactones: a new class of hepatitis A virus 3C cysteine proteinase inhibitors." J Org Chem **67**(5): 1536-47.
- Landry, M. L., S. Stanat, et al. (2000). "A standardized plaque reduction assay for determination of drug susceptibilities of cytomegalovirus clinical isolates." Antimicrobial Agents & Chemotherapy **44**(3): 688-92.
- Lawson, M. A. and B. L. Semler (1990). "Picornavirus protein processing—enzymes, substrates, and genetic regulation." Curr Top Microbiol Immunol **161**: 49-87.
- Levine, L. M., M. L. Michener, et al. (1997). "Measurement of specific protease activity utilizing fluorescence polarization." Anal Biochem **247**(1): 83-8.
- Lipinski, C. A., F. Lombardo, et al. (2001). "Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings." Adv Drug Deliv Rev **46**(1-3): 3-26.
- Malcolm, B. A. (1995). "The picornaviral 3C proteinases: cysteine nucleophiles in serine proteinase folds." Protein Sci **4**(8): 1439-45.
- Margosiak, S. A., D. L. Vanderpool, et al. (1996). "Dimerization of the human cytomegalovirus protease: kinetic and biochemical characterization of the catalytic homodimer." Biochemistry **35**(16): 5300-7.
- Peters, H., Y. Y. Kusov, et al. (2005). "Hepatitis A virus proteinase 3C binding to viral RNA: correlation with substrate binding and enzyme dimerization." Biochem J **385**(Pt 2): 363-70.
- Pinko, C., S. A. Margosiak, et al. (1995). "Single-chain recombinant human cytomegalovirus protease. Activity against its natural protein substrate and fluorogenic peptide substrates." Journal of Biological Chemistry **270**(40): 23634-40.
- Pray, T. R., K. K. Reiling, et al. (2002). "Conformational change coupling the dimerization and activation of KSHV protease." Biochemistry **41**(5): 1474-82.

- Qiu, X., J. S. Culp, et al. (1996). "Unique fold and active site in cytomegalovirus protease." Nature **383**(6597): 275-9.
- Roehm, N. W., G. H. Rodgers, et al. (1991). "An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT." J Immunol Methods **142**(2): 257-65.
- Schindler, T., W. Bornmann, et al. (2000). "Structural mechanism for STI-571 inhibition of abelson tyrosine kinase." Science **289**(5486): 1938-42.
- Shieh, H. S., R. G. Kurumbail, et al. (1996). "Three-dimensional structure of human cytomegalovirus protease.[erratum appears in Nature 1996 Nov 21;384(6606):288]." Nature **383**(6597): 279-82.
- Shimba, N., A. M. Nomura, et al. (2004). "Herpesvirus protease inhibition by dimer disruption." Journal of Virology **78**(12): 6657-6665.
- Stahle, E. L., L. Schloss, et al. (1998). "Solid phase ELISA for determination of the virus dose dependent sensitivity of human cytomegalovirus to antiviral drugs in vitro." Antiviral Research **40**(1-2): 105-12.
- Tong, L., C. Qian, et al. (1996). "A new serine-protease fold revealed by the crystal structure of human cytomegalovirus protease." Nature **383**(6597): 272-5.
- Welch, A. R., A. S. Woods, et al. (1991). "A herpesvirus maturational proteinase, assemblin: Identification of its gene, putative active site domain, and cleavage site." Proc Natl Acad Sci U S A **88**: 10792-10796.
- Yoakim, C., W. W. Ogilvie, et al. (1998). "Potent beta-lactam inhibitors of human cytomegalovirus protease." Antivir Chem Chemother **9**(5): 379-87.
- Zhang, J. H., T. D. Chung, et al. (1999). "A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays." J Biomol Screen **4**(2): 67-73.

5. Figures

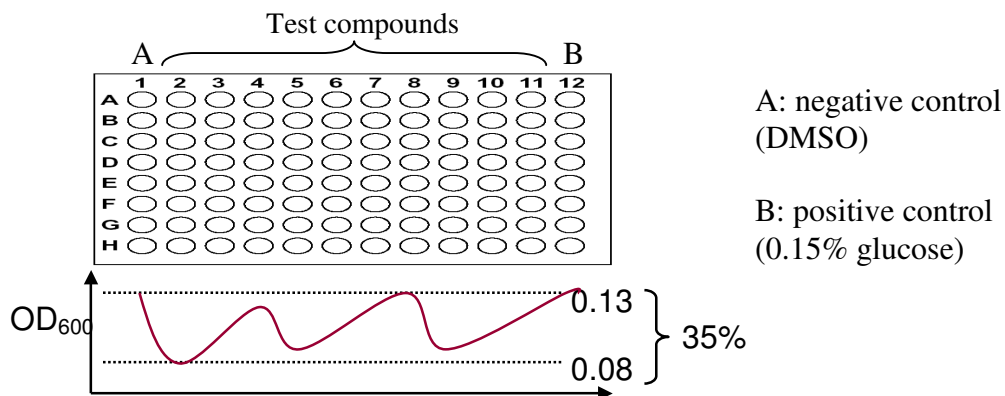


Figure 1. Illustration of OD₆₀₀ variations between the columns of a microtiter plate when glycerol and 3-AT are used.

Table I

	Hand optimized LexA-M assay	Automated LexA-M assay
Substrate promoter	<i>ADHtr</i> (<i>TRP1</i> marker)	<i>ADHtr</i> (<i>TRP1</i> marker)
Protease promoter	<i>ACT4</i> (<i>URA3</i> marker)	<i>GAL1</i> (<i>LEU2</i> marker)
Medium	-HUT 2% glu + 10% glycerol + 10 mM 3-AT	-HLT 2% gal
Inoculation OD ₆₀₀	0.01	0.001
OD ₆₀₀ difference between the columns	35%	5%
Maximal fold stimulation	4x	1.6x
Z' factor	0.62	0.54

Figure 2 (next page). Overview of the experimental procedure for the screening and characterization of HCMV protease inhibitors.

Figure 2

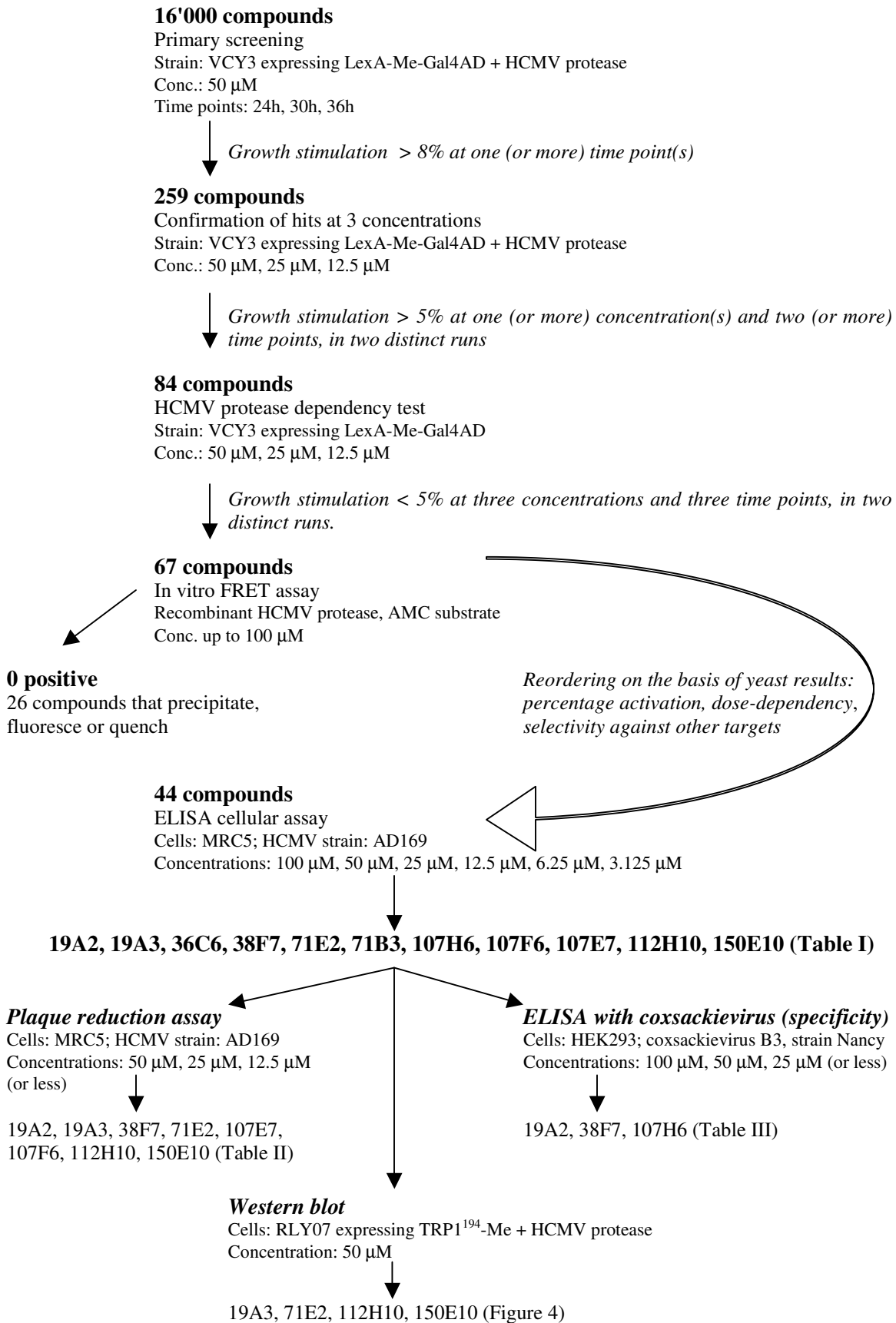


Table II

Anti-HCMV activity and cytotoxicity of selected compounds			
Compound	IC ₅₀ in ELISA ^a	IC ₅₀ in PRA ^a	% toxicity ^b at 100 µM
GCV	0.62 µM	3 µM	0%
BI36	60 µM	ND	6%
19A2	53 µM	10 µM	25%
19A3	34.5 µM	> 12.5 µM*	18%
36C6	51 µM	> 250 µM	20%
38F7	10 µM	30 µM	22%
71E2	73.2 µM	83 µM	0%
71B3	> 25 µM*	ND	18%
107E7	43 µM	50 µM	0%
107F6	30 µM	> 3 µM*	80%
107H6	23 µM	ND	60%
112H10	60 µM	95 µM	0%
150E10	8 µM	22 µM	80%

^a IC₅₀: 50% inhibitory concentration

^b toxicity of compounds on MRC5 cells is measured by XTT assay

* higher concentration tested; compounds precipitated or showed >20% toxicity above

Table III

Anti-CVB3 activity and cytotoxicity of selected compounds		
Compound	IC ₅₀ in ELISA ^a	% toxicity ^b at 100 µM
GCV	> 100 µM	0%
BI31	13.2 µM	25%
19A2	> 100 µM	10%
19A3	55 µM	25%
36C6	90 µM	10%
38F7	> 100 µM	0%
71E2	63.7 µM	10%
71B3	> 25 µM*	20%
107E7	> 25 µM*	50%
107H6	> 100 µM	10%
112H10	40 µM	25%
150E10	8.4 µM	70%

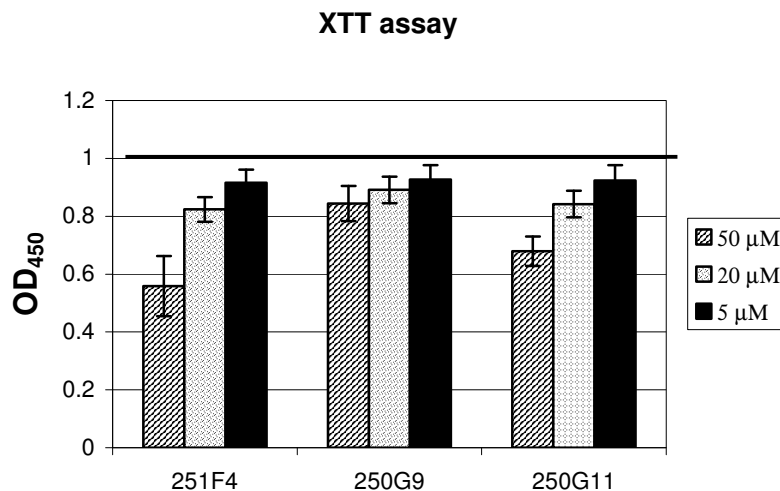
^a IC₅₀: 50% inhibitory concentration

^b toxicity of compounds on HEK293 cells is measured by XTT assay

* higher concentration tested; compounds precipitated or showed > 20% toxicity above

In bold: compounds that do not inhibit coxsackievirus

A.



B.

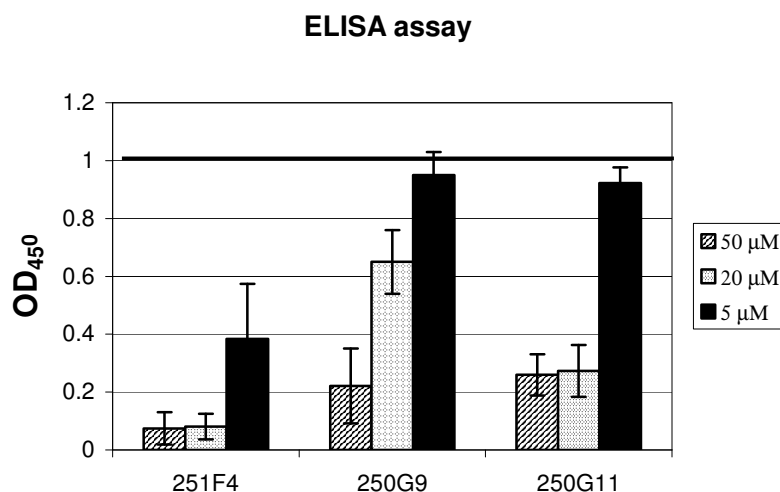


Figure 3. Unspecific, toxic compounds prevent HCMV proliferation in cell culture. A. Compound toxicity on MRC5 cells, as measured by XTT assay. The assay was made 7 days after cell seeding and 5 days after the treatment with the compounds. B. Compound efficiency against HCMV, as measured by ELISA assay. Assay was performed 7 days after cell seeding and infection with HCMV, and 5 days after adding the compounds.

Table IV**Hit validation in yeast: LexA-M system with 10% glycerol**

Compound	Fold stimulation against active protease at 50 μ M	Fold stimulation against inactive protease at 50 μ M
BI31	1.27x	0.61x
BI36	1.94x	0.86x
19A2	0.95x	0.77x
19A3	1.06x	1.1x
36C6	1.18x	1.11x
38F7	1.1x	1.08x
71E2	0.86x	1x
71B3	ND	ND
107E7	1.10x	0.96x
107H6	1.06x	1.05x
112H10	1.06x	1.02x
150E10	1.12x	1.02x

In bold: compounds that would have been scored as positives in the primary screen if this assay would have been used.

Z' factor: 0.62

Maximal fold stimulation in case of total inhibition of active protease: 2.7x

Table V**Hit validation in yeast: Trp1-M system with 10% glycerol**

Compound	Fold stimulation against active protease at 50 μ M	Fold stimulation against inactive protease at 50 μ M
BI31	3,75x	0.61x
BI36	6,1x	0.93x
19A2	0.92x	0.80x
19A3	5.44x	0.82x
36C6	1.16x	0.98x
38F7	0.54x	0.85x
71E2	0.63x	0.96x
71B3	0.4x	0.83x
107E7	1.10x	0.73x
107H6	1.06x	0.97x
112H10	1.36x	0.97x
150E10	3.78x	1.16x

In bold: compounds that would have been scored as positives in the primary screen if this assay would have been used.

Z' factor: 0.85

Maximal fold stimulation in case of total inhibition of active protease: 10x

Table VI**Activity of BI compounds in LexA-M system in absence of glycerol at 36h**

Compound	Fold stimulation against active protease at 50 μ M	Fold stimulation against inactive protease at 50 μ M
BI31	0.77x	0.60x
BI35	1.05x	0.78x
BI36	1.14x	0.87x

In bold: compounds that would have been scored as positives in the primary screen

Z' factor: 0.54

Maximal fold stimulation in case of total inhibition of active protease: 1.6x

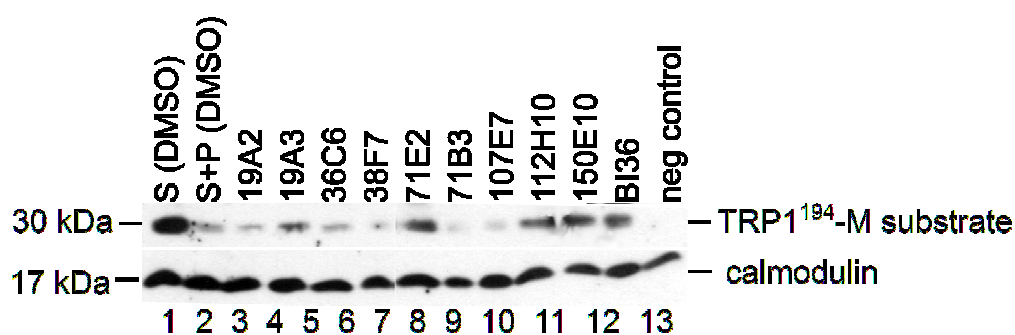


Figure 4. Western blot analysis of yeast extracts to test whether hits prevent cleavage of the Trp1¹⁹⁴-Me substrate by HCMV protease. RLY07 cells were transformed with Trp1¹⁹⁴-Me alone (lane 1), Trp1¹⁹⁴-Me and active protease (lane 2-12), or 2 empty plasmids (lane 13). Transformed cells were incubated in growth medium with the different hits at 50 μ M (lane 3-11), with BI36 control at 50 μ M (lane 12) or with DMSO (lane 1,2,13). The calmodulin antibody served as an internal control for protein amount.

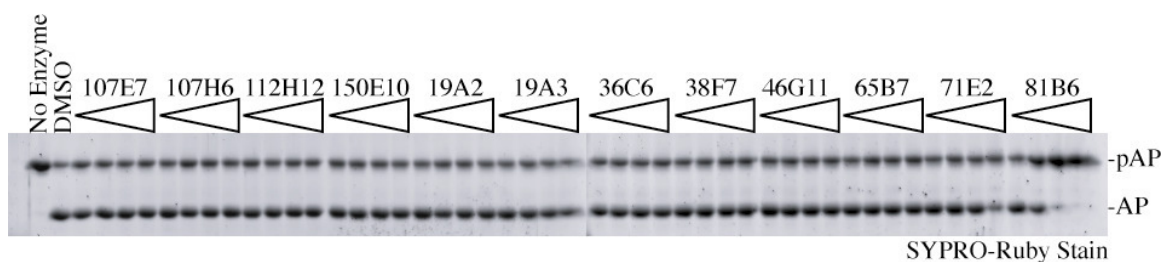
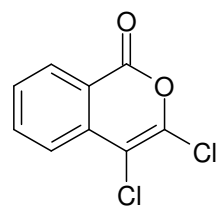
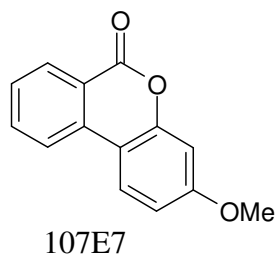


Figure 5. *In vitro* analysis of compound effect on pAP cleavage by HCMV protease. 1 μ M pAP substrate was incubated with 100 nM assemblin and different concentrations of compounds (0.1 μ M, 1 μ M, 10 μ M, 100 μ M) in assay buffer during 1 hour at RT. Cleavage was then addressed by SDS-PAGE.

A



B

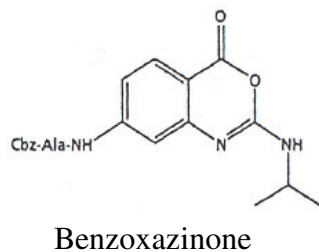
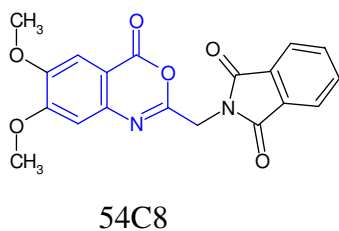


Figure 6: Comparison between hits (left) and published HCMV protease inhibitors (right).

Table VII (next page):

a: fold stimulation at 50 μ M

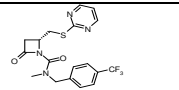
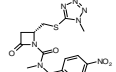
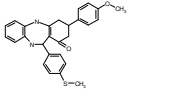
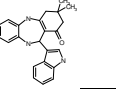
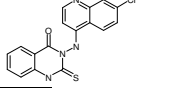
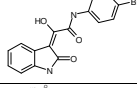
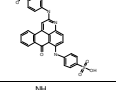

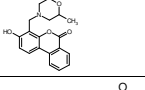
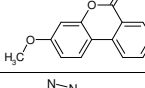
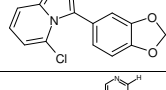

b: visual estimation of inhibition at 50 μ M

c: IC₅₀: 50% inhibitory concentration

d: % toxicity at 100 μ M; toxicity is measured by XTT assays.

* highest concentration tested; compounds precipitated or showed >10% toxicity above.

In bold: characteristics expected from an HCMV protease inhibitor.

		Yeast data							<i>In vitro</i> data		Mammalian cells data				
Compound	Structure	LexA-M system (automated) ^a		LexA-M system (glycerol) ^a		TRP1-M system (glycerol) ^a		Western blot ^b	FRET assay ^c	SDS-PAGE (Gibson) ^b	ELISA assay ^c (HCMV)	PRA assay ^c (HCVM)	Toxicity on MRC5 cells ^d	ELISA assay ^c (CV)	Toxicity on HEK293 cells ^d
		HCMV protease	No protease	HCMV protease	No protease	HCMV protease	No protease								
BI31		0.77x	0.60x	1.27x	0.61x	3,75x	0.61x	ND	1.8 μM	ND	70 μM	ND	15%	13.2 μM	25%
BI36		1.15x	0.87x	1.94x	0.86x	6,1x	0.93x	+++	0.3 μM	+++	60 μM	ND	6%	ND	ND
19A2		1.08x	0.84x	0.95x	0.77x	0.92x	0.80x	-	quenching	-	53 μM	10 μM	25%	> 100 μM	10%
19A3		1.26x	1.23x	1.06x	1.10x	5.44x	0.82x	+	quenching	-	60 μM	> 12.5 μM*	18%	55 μM	25%
36C6		1.08x	0.88x	1.18x	1.11x	1.16x	0.98x	-	quenching	-	51 μM	> 250 μM	20%	90 μM	10%
38F7		1.19x	1.00x	1.10x	1.08x	0.54x	0.85x	-	quenching	-	10 μM	30 μM	10%	> 100 μM	0%
71E2		1.20x	0.90x	0.86x	1.00x	0.63x	0.96x	+++	quenching	-	65 μM	83 μM	0%	63.7 μM	10%
71B3		1.13x	0.92x	ND	ND	0.40x	0.83x	ND	quenching	-	> 25 μM*	ND	18%	> 25 μM*	20%
107H6		1.09x	0.85x	1.06x	1.05x	1.06x	0.97x	ND	fluo	-	30 μM	> 3 μM*	80%	> 100 μM	10%
107E7		1.15x	0.99x	1.10x	0.96x	1.10x	0.73x	-	fluo	-	43 μM	50 μM	0%	> 25 μM*	50%
112H10		1.15x	1.00x	1.06x	1.02x	1.36x	0.97x	+++	-	-	60 μM	95 μM	0%	40 μM	25%
150E10		1.27x	0.95x	1.12x	1.02x	3.78x	1.16x	+++	fluo	-	8 μM	22 μM	80%	8.4 μM	70%

4. General conclusion

Successful application of protease inhibitors in human therapy requires defined properties of drugs, such as membrane permeability, stability and lack of toxicity (Barberis 2002). While most HTS campaigns are performed with enzymatic *in vitro* assays, where compounds are selected exclusively in regard of their potential to inhibit proteolytic activity, cellular screening systems provide a promising alternative to select directly for compounds with additional drug-like features. Indeed, compounds identified as hits in living cells not only should inhibit proteolytic activity, but must also be stable within the cell, capable of penetrating biological membranes, and exert no or only limited toxic effects on the cell.

In this thesis, two yeast growth selection systems were developed to identify HCMV protease inhibitors: the LexA-M and the Trp1-M systems. The LexA-M system is based on other studies, which took advantage of the two functionally separable essential domains of Gal4p to measure proteolytic activity, either in mammalian cells (Lawler and Snyder 1999) or in yeast (Dasmahapatra, DiDomenico et al. 1992; Murray, Hung et al. 1993). In these studies, the inactivation of the Gal4p transcription factor by a protease caused stop of transcription of a Gal4p regulated reporter gene. In our yeast system, HCMV protease similarly cleaves the M-site inserted between the two domains of Gal4p, and thus prevents transcription of the *HIS3* reporter gene. As described in Part I, the optimization of the LexA-M system was a long process, and several key steps were necessary to improve HCMV protease efficiency in yeast, namely the replacement of the full-length protease precursor by the assemblin catalytic domain, the addition of glycerol in the selection medium and the elongation of the original 13 amino acid cleavage site to a 39 amino acid sequence.

In Part II of this thesis, an alternative system was developed, which was based on a new concept but benefited from the experience accumulated during the development of the LexA-M system. In this Trp1-M system, the HCMV protease cleavage sequence is inserted into a yeast protein essential for cell proliferation, i.e. the yeast marker Trp1p. Cleavage and subsequent functional inactivation of Trp1p cause conditional growth arrest. Here like in the LexA-M system, protease activity correlates inversely with cell growth. Inhibition of the protease by small molecules stimulates the proliferation of the

cells. Thus, both systems are based on positive read-out and therefore select for non toxic compounds. HCMV protease inhibitors developed by Boehringer Ingelheim were used as positive control to validate these two systems (see II.3.5 and III.2.6).

Both systems are equivalent in terms of sensitivity. I calculated for BI36 IC_{50} values of 35 μ M and 31 μ M in the LexA-M and Trp1-M assays, respectively. However, the risk of selecting false positives is reduced in the Trp1-M system since the pathway leading to growth inhibition is shorter. Indeed, in this assay only compounds that interact with expression of the substrate or of the protease can show up as positives, whereas in the LexA-M system compounds might also interact with LexA dimerization, LexA binding to the DNA, *HIS3* transcription, synthesis of His3p enzyme, activity of His3p enzyme. Thus, the Trp1-M system is probably more reliable. In any case, since the two assays are based on different mechanisms a compound scored as positive in both assays has a good chance to be a real positive.

In this thesis, both systems are described as tools for the identification of HCMV protease inhibitors. But their use can be broadly extended. Screening for inhibitors should be possible for most viral proteases, like for example other herpesvirus proteases, picornavirus proteases, HIV protease, and also for human soluble proteases, like for instance caspases, cathepsins, calpains. For that matter, coxsackievirus 3C and varicella-zoster virus proteases were expressed with their corresponding hybrid substrate in LexA-“M” system, and they inhibited cell proliferation as expected (data not shown). The same was observed for coxsackievirus protease 3C in Trp1-“M” system (data not shown). Thus, our systems can be easily adapted for monitoring the activity of proteases other than HCMV protease. Moreover, they could theoretically also be used to identify substrates of orphan proteases, or conversely, proteases responsible for the cleavage of given substrates. Alternatively, a protease mutagenesis study could be made to increase the activity of a protease or to change its substrate specificity, like it was done by the DIREVO researchers, who engineered a standard protease so that it cleaves selectively TNF α (<http://www.direvo.de/press.html>; press release of October 20th).

In Part III, the LexA-M system was applied to a high-throughput screening for identification of HCMV protease inhibitors. The screening of 15'000 small molecules delivered 67 potential inhibitors, which counteracted the growth inhibition caused by HCMV protease. Among them, 8 compounds showed good antiviral properties against

HCMV in both ELISA and PRA mammalian cell assays. It was not clear though, if this effect was due to the inhibition of the viral protease or to the toxicity of the compounds. To address this question compounds were tested first in *in vitro* FRET assays, where they were either inactive or could not be investigated because of their fluorescence or quenching properties (Table VII). Next, their influence on the cleavage of the TRP1¹⁹⁴-M substrate in yeast was evaluated by Western blot. However, this experiment did not allow discriminating between *bona fide* inhibitors and compounds that would modify substrate or protease expression. Finally, the capacity of compounds to prevent the cleavage of the natural substrate pAP *in vitro* was examined. In this assay performed in the laboratory of Professor Gibson (Johns Hopkins University School of Medicine, Baltimore) none of the hits was able to prevent cleavage. However, as discussed in III.3, it is not excluded that our compounds inhibit dimerization of HCMV protease. In this case, hits might be inactive in biochemical assays because of the high salt concentration that is usually used in the buffers to force the dimerization and so keep the enzyme in an active conformation.

Several reasons can explain why no inhibitor has been identified, which would have been active not only in yeast and mammalian cell assays, but also in biochemical assays. First, it has to be reminded that HCMV protease is a difficult target. Intensive researches have been carried out since more than 10 years to develop protease inhibitors, and so far to our knowledge none of them entered clinical trials (Waxman and Darke 2000; Borthwick 2005). The difficulty with HCMV protease resides in the shallowness of its active site (Chen, Tsuge et al. 1996). As a result, chemical compounds have few possibilities to bind to the active site, and it is therefore difficult to design selective inhibitor. It is also the reason why most inhibitors published so far bind covalently to the enzyme (Deziel and Malenfant 1998; Borthwick, Exall et al. 2002; Baum, Ding et al. 1996). As it is discussed in III.3, an elegant alternative to the active site inhibition would be the dimer disruption of the enzyme, which would also inhibit proteolytic activity. Such dimerization inhibitors would probably show a higher degree of specificity compared to active site inhibitors.

A second argument to explain the lack of convincing inhibitors is the limited size of the library. Indeed, our library consists only of 15'000 compounds, which is far away from the typical multimillion compound libraries of pharmaceutical companies (Coburn, Stachel et al. 2004).

And finally, as discussed in chapter III.2.1 and III.2.7, the conditions applied for HTS screening were not optimal. To ensure a homogeneous cell culture pipetting by the robot in microtiter plates, glycerol was removed from the selecting medium, whereas it was shown that this chemical greatly improves protease efficiency (I.2.4). As a consequence the sensitivity of the LexA-M system was reduced. Therefore, if a new screening would be performed we would search another solution to solve the “wavy pattern” problem.

In summary, even if encouraging results were obtained for several compounds in different assays (150E10 for example is very potent in yeast and antiviral assays, and seems to inhibit cleavage in the Western blot), no evidence of direct inhibition of HCMV protease could be produced. For each compound, the data collected present discrepancies (see Table VII): some compounds were active in one or two yeast assay(s), but not in the other(s); for many of them, no reduction of cleavage was observed in Western blot; in mammalian cell assays, most compounds were not specific and inhibited both HCMV and CV. Thus, considering in particular the lack of evidence of protease inhibition by our hits in biochemical assay, it has been decided to not develop these hits further.

References

- Barberis, A. (2002). "Cell-based high-throughput screens for drug discovery." European BioPharmaceutical Review(winter).
- Baum, E. Z., W. D. Ding, et al. (1996). "Flavins inhibit human cytomegalovirus UL80 protease via disulfide bond formation." Biochemistry **35**(18): 5847-55.
- Borthwick, A. D. (2005). "Design of transactam HCMV protease inhibitors as potent antivirals." Med Res Rev **23**: 23.
- Borthwick, A. D., A. M. Exall, et al. (2002). "Pyrrolidine-5,5-trans-lactams as novel mechanism-based inhibitors of human cytomegalovirus protease. Part 3: potency and plasma stability." Bioorg Med Chem Lett **12**(13): 1719-22.
- Chen, P., H. Tsuge, et al. (1996). "Structure of the human cytomegalovirus protease catalytic domain reveals a novel serine protease fold and catalytic triad." Cell **86**(5): 835-43.
- Coburn, C. A., S. J. Stachel, et al. (2004). "Identification of a small molecule nonpeptide active site beta-secretase inhibitor that displays a nontraditional binding mode for aspartyl proteases." J Med Chem **47**(25): 6117-9.
- Dasmahapatra, B., B. DiDomenico, et al. (1992). "A genetic system for studying the activity of a proteolytic enzyme." Proc Natl Acad Sci U S A **89**(9): 4159-62.
- Deziel, R. and E. Malenfant (1998). "Inhibition of human cytomegalovirus protease N(o) with monocyclic beta-lactams." Bioorg Med Chem Lett **8**(11): 1437-42.
- Lawler, J. F., Jr. and S. H. Snyder (1999). "Viral protease assay based on GAL4 inactivation is applicable to high- throughput screening in mammalian cells." Anal Biochem **269**(1): 133-8.
- Murray, M. G., W. Hung, et al. (1993). "Inactivation of a yeast transactivator by the fused HIV-1 proteinase: a simple assay for inhibitors of the viral enzyme activity." Gene **134**(1): 123-8.
- Waxman, L. and P. L. Darke (2000). "The herpesvirus proteases as targets for antiviral chemotherapy." Antiviral Chemistry & Chemotherapy **11**(1): 1-22.

5. Appendix

5.1. Yeast plasmids and promoters

CEN4-ARS1 versus 2 micron (2 μ):

CEN4-ARS1 plasmid: single copy plasmid (=> low intracellular protein concentration)

2 micron (2 μ) plasmid: high copy plasmid (=> high intracellular protein concentration)

Level of protein expression with following promoters and plasmids:

(*LacZ* reporter gene was cloned under the following promoters, and expression of β -galactosidase was normalized to *Gal1* promoter)

<u>Promoter</u>	<u>Plasmid (ori)</u>	<u>β-gal value</u>
<i>ADH_{tr}</i> : truncated <i>ADH1</i>	CEN4-ARS1	0.8 %
<i>ADH_{fl}</i> : full-length <i>ADH1</i>	CEN4-ARS1	2.5 %
<i>ADH_{tr}</i> : truncated <i>ADH1</i>	2 μ	5 %
<i>Gal1</i> truncated 7%	CEN4-ARS1	7 %
<i>ACT4</i>	CEN4-ARS1	8.5 %
<i>Gal1</i> truncated 16%	CEN4-ARS1	16 %
<i>Gal1</i> truncated 46%	CEN4-ARS1	46 %
<i>Gal1</i> truncated 71%	CEN4-ARS1	71 %
<i>ADH_{fl}</i> : full-length <i>ADH1</i>	2 μ	95 %
<i>Gal1_{fl}</i>	CEN4-ARS1	100 %
<i>ACT4</i>	2 μ	250 %

5.2. Abbreviations

HCMV: Human cytomegalovirus

CV: Cocksackievirus

VZV: Varicella-zoster virus

HSV1: Herpes simplex virus I

pAP: Assembly protein precursor (natural substrate of HCMV protease)

pPR: Protease precursor (immature form of HCMV protease)

PFU: Plaque forming unit; number of infectious virus particles per unit volume.

MOI: Multiplicity of infection; number of infectious virus particles per cell in a specific experiment.

CPE: Cytopathic effect; symptom of viral infection in a cell culture.

6. Acknowledgements

I would like to thank Alcide Barberis for his supervision, his advices and his motivating support; Dominik Escher for offering me the opportunity to perform my Ph.D. thesis at ESBATech and to participate to scientific conferences. I am also grateful to Prof. Fritz Müller for having accepted me as his first external Ph.D. student.

I thank Dr. Christiane Yoakim for her efforts to press the legal department of Boeringher Ingelheim (tough mission!) so that I could receive the BI31, BI35 and BI36 compounds and later send my paper for publication. It was good to talk to her and to hear the accent from Quebec! I also thank Professor Wade Gibson for spending time in testing my compounds.

Further I would like to thank Urs, who as the leader of the protease unit was always ready to help, to answer questions and to give unbiased feedback. I also thank Karin, who was a precious support for “serious stuff” (plasmid maps, protocols, inoculation of colonies on Sunday night...) as well as “less serious stuff” (event organization, returning home after too many caipirinhas...). I am grateful to Eva Loepfe (from the Institute of Medical Virology at the University of Zürich) for introducing me to virus culture. I thank Viola for introducing me to cell culture, for her “Go, go Valerie, Go!” and her permanent good mood; Julia and Susanne for their encouragements and their interest in my work; Greg for always rescuing me when I was fighting with my computer; Stefan B. for initiating me to heavy metal music (even if I am not totally convinced yet), Cathy, Leo, Tea, David, Anita, Barbara, Oli, Claudia, Adi, Michi, Stephan A., Stefan K., Isabelle, Maya, Mix, Simone, Daniela, Peter S., Peter L., Werner... all those who contributed one way as the other to my work.

Finally, I thank my parents and my friends for their support during these four years.

7. Attachments

7.1 Curriculum vitae

Nom	Cottier
Prénom	Valérie
Date de naissance	9 avril 1975
Lieu d'origine	Jaun (FR)

Formation

1990-1994 Collège de Bulle, Maturité Type B (latin)

1995-2000 Etudes de biologie, Université de Fribourg
Branche principale : Biologie moléculaire et cellulaire des plantes
Branche secondaire : Physiologie
Branche complémentaire : Biologie du développement

Travail de diplôme: *“Influence de l’acide β -amino-butyrique sur la susceptibilité d’Arabidopsis à différents pathogènes et sur sa fertilité”*

1998-2000 Diplôme de Maîtresse de Gymnase

2002-2006 Thèse de doctorat dans l’entreprise de biotechnologie ESBATech AG, à Schlieren, sous la direction du Prof. Dr. Fritz Müller (Institut de biologie développementale et cellulaire de l’Université de Fribourg) et du Dr. Alcide Barberis (ESBATech AG).

Titre de la thèse: *“Yeast growth selection system for the identification of cell-active inhibitors of human cytomegalovirus protease”*

7.2. Publications

1. Cottier V., A. Barberis and U. Luethi (2006). "A novel yeast cell-based assay to screen for inhibitors of human cytomegalovirus protease in a high-throughput format." Antimicrob Agents Chemother. **50**(2): 565-571
2. Jakab G., V. Cottier, V. Toquin, G. Rigoli, L. Zimmerli, J.P. Métraux and B. Mauch-Mani (2001). "β-aminobutyric acid-induced resistance in plants." Eur J Plant Pathol **107**: 29-37

7.3. Patent

Cottier V., A. Barberis, U. Lüthi. "Genetic selection system to identify proteases, protease substrates and protease inhibitors." ESBATech AG. International application published under the patent cooperation treaty (PCT). *Manuscript submitted.*

7.4. Conferences

1. *Proteolytic Enzymes & Their Inhibitors* (GRC). New London, NH, USA, July 2004. Poster presentation.
2. *Swiss Yeast Meeting*. Fribourg, September 2004. Presentation: "HTS in Yeast for the Identification of Cell-Active Inhibitors of Cytomegalovirus Protease"