Master Thesis

Improvements of a methodology for functional inactivation of proteases in apicomplexan parasites by using natural or molecular engineered serpins

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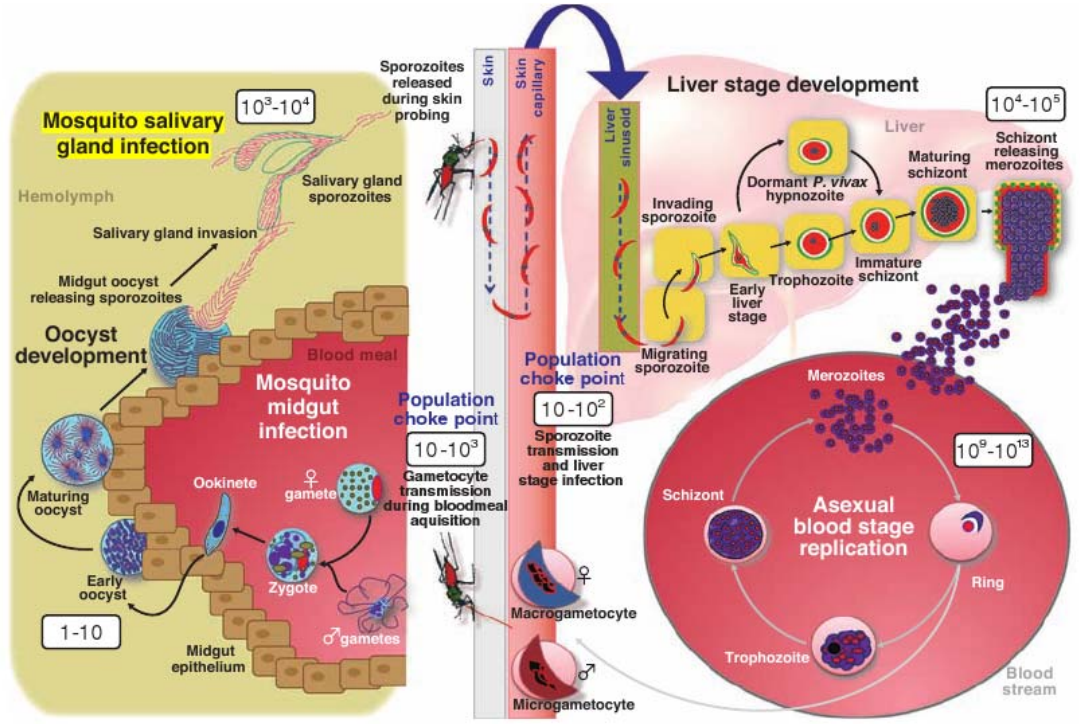
Introduction

Malaria is a catastrophic disease that results in 800,000 deaths annually (1). More than a 40% of global population is at a high risk to get malaria infection. The causative agents of malaria belong in the genus Plasmodium and they have a complex life cycle. When the mosquito bites a human, the parasite transfers from skin to the capillaries and from there, it enters the bloodstream. The parasite transfers with the bloodstream to the liver and finally enters the liver cells through the sinusoids. The sporozoite will undergo a rapid proliferation and at the end of the process many merozoites will be released to the bloodstream. These merozoites will infect the red blood cells. In the red blood cells, the parasite can undergo two different routes. Sequential differentiation into ring, trophozoite, and finally schizont/merozoite stages, or alternatively differentiation into macro- and micro-gametocytes.

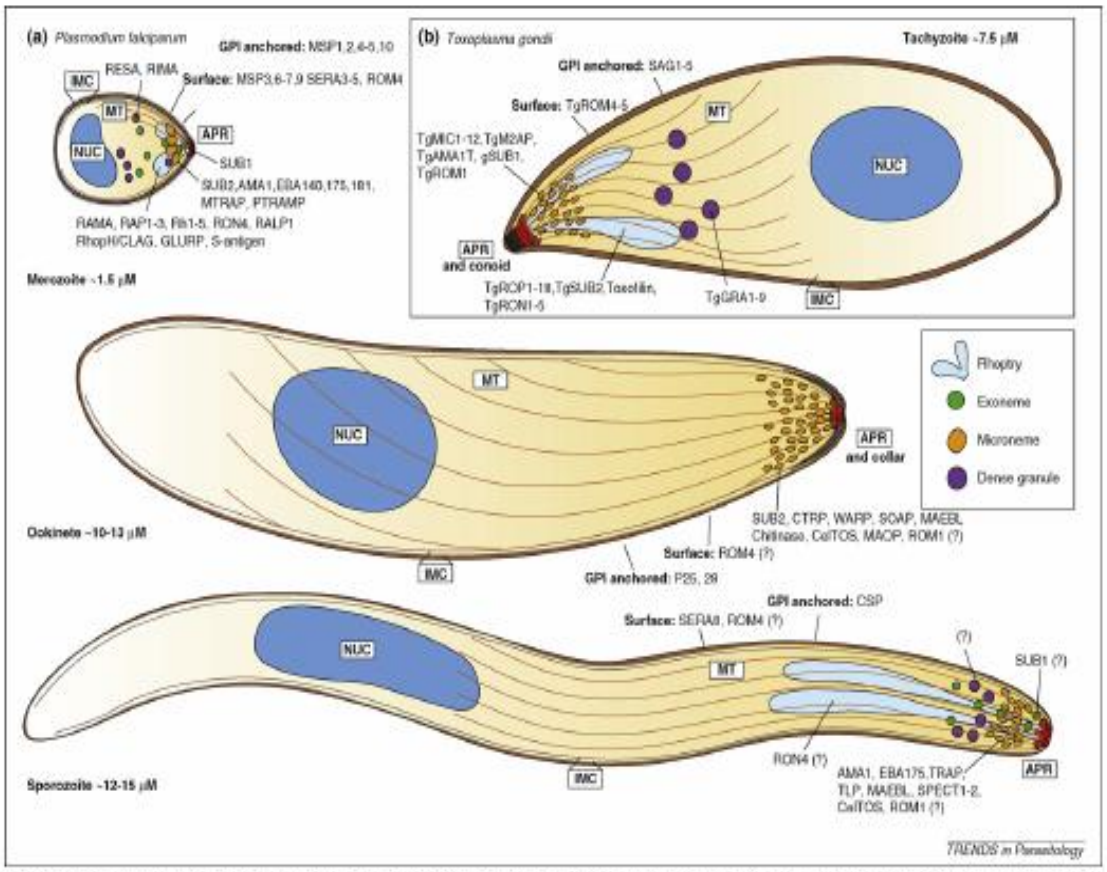
In the midgut of mosquito, gametocytes will further develop into gametes that will fuse to form a zygote. The zygote will differentiate into an ookinete. The ookinete will traverse the mosquito midgut epithelium and will differentiate into an oocyst at the basal side of the midgut. The oocyst ruptures and the released sporozoites invade the salivary glands of the mosquito. The sporozoites will enter the bloodstream of healthy human with a new mosquito bite initiating a new disease cycle.



Picture 1. Science 328.862 (2010)

All the apicomplexa including Plasmodium contain a series of specific sub-cellular organelles. These organelles have distinct functions but altogether they mediate the processes of invasion, egress and remodeling of the host cell. Rhoptries and micronemes are essential for the invasion process (2) while dense granules are essential for the formation of the parasitophorous vacuole and possibly host cell manipulation (2). Micronemes are the only invasion related organelles in ookinete, the stage that invades mosquito midgut epithelium. Exonemes mediate the remodeling of the host cell and were recently found in merozoites and sporozoites (2). Exonemes were not so far identified in the other

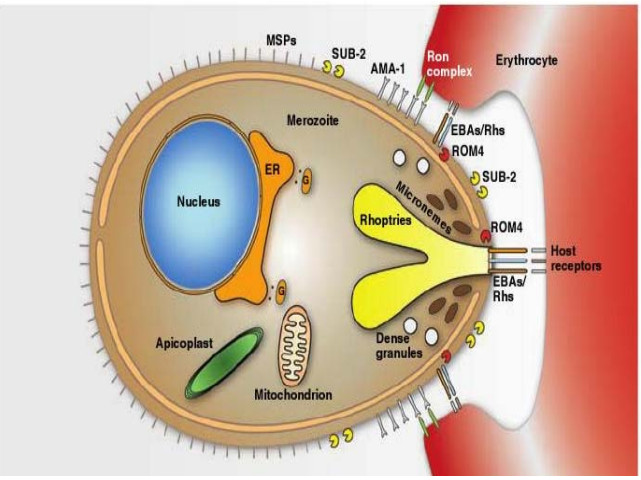
important human pathogen *Toxoplasma gondii* but all the other organelles are present.



Picture 2: The left part of the picture demonstrates the different stages of the Plasmodium berghei. From top to bottom one can observe the differences between the merozoite, the ookinete and the sporozoite. The upper right part of the schematic termed (b) illustrates the parasite Toxoplasma gondii. Nuc stands for nucleus. Rhoptries are shown in light blue, exonemes are shown in green, micronemes are depicted as orange and finally dense granules have dark blue color. Trends in Parasitology doi:10.1016/j.pt.2008.08.006

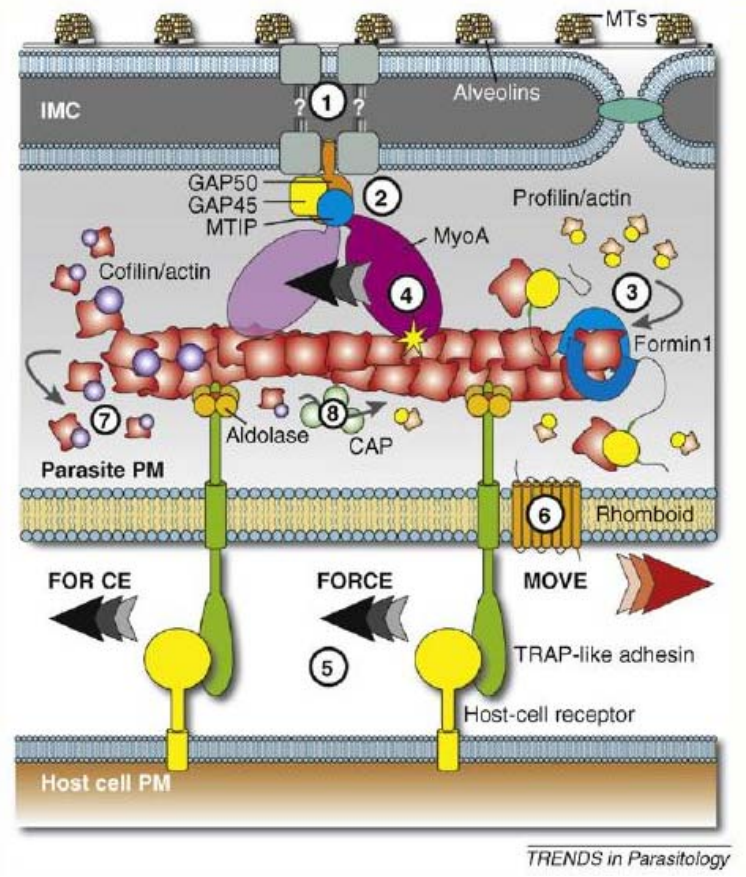
PARASITE INVASION-EGRESS AND PROTEASES

Important roles during invasion of apicomplexan parasites are performed by surface associated adhesins and proteases. There are certain adhesins at the parasite's cell surface, which associate with host cell receptors (i.e. AMA-1, EBA). In order for the parasite to invade, the host cell receptors must dissociate from adhesins and associate again with adhesins that reside more posteriorly. The role of intra-membrane (*rhomboid: ROM4*, depicted as red), and membrane associated (*subtilisin: SUB2,* depicted as yellow), proteases is to cleave adhesins either within the intra-membrane part or close to the membrane respectively (juxtamembrane positions) (3,4).



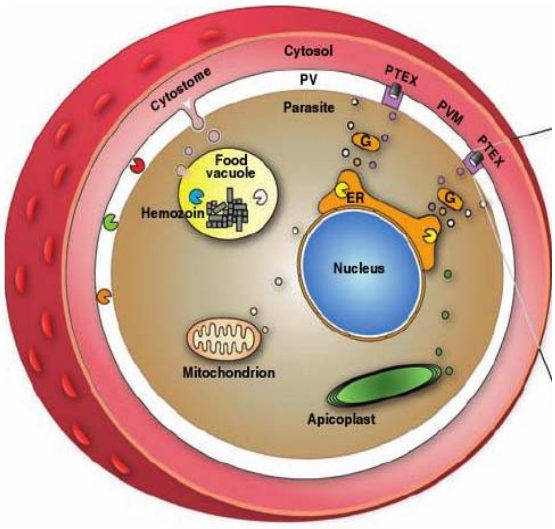
Picture 3: The schematic illustrates the invasion of the merozoite into the erythrocyte. SUB2 protease is depicted in a yellow "pacman" like figure and the ROM4 protease is depicted in red "pacman" like figure. The "pacman" figure exemplifies the proteolytic activity of the proteases. Adhesins of the merozoite, depicted as light blue are shown to associate with the receptors of the erythrocyte. Science 328.862 (2010)

Lately, the mechanism by which association of host cell receptors with adhesins results into the movement of the parasite started to be elucidated. Step 1 shows the existence of specific sub-cellular organelles called intra membrane cisternae (hereafter termed IMC). Step 2: The IMC attach with one side to the alveolins and with the other side to the GAP50 (5). GAP50, GAP45 and microtubule light chain MTIP associate with Myosin A (6, 7, 8). Step 3: Profilin and formin are responsible for the polymerization of the G-actin molecules to F-actin (9, 10). Step 4: "Myosin heads" move along the polymerized actin in an ATP-dependent manner. Step 5: The forward movement of the F actin propels the TRAP -like adhesin and this results in the movement of the parasite towards the direction of the red arrow. Step 6: ***Intra-membrane proteases*** **cleave the trans-membrane regions of adhesins (3).** Step7: Cofilin and CAP severe the actin molecules from the F-actin (12).



Picture 4 Notice the trans-membrane region of the Rhomboid protease that enables it to cleave the TRAP like adhesin in its trans-membrane region. Although adhesins demonstrate high polymorphism, the key position of the rhomboid in the plasma membrane renders it a beautiful drug target. Red arrows depict the direction of movement and black arrows depict the direction of the exertion of force. Trends in Parasitology doi:10.1016/j.pt.2008.0806

At the end of merozoite differentiation/growth within the red blood cells, egress of the parasite is initiated. With the term egress is considered the breakdown of the parasitophorous vacuole membrane (hereafter termed PVM), within which the parasite resides and differentiates and which is formed primarily from the parasite and of the plasma membrane of the RBC.



Picture 5: The egress is a proteolytic cascade in which a major role is played by three proteases termed Subtilisin1:SUB1. DPAP1 and SERA5, depicted as red, green and orange respectively (13). Science 328, 862 (2010)

Most of the apicomplexan proteases that participate into parasite invasion and egress are essential molecules. Essential are considered molecules whose genetic disruption would result into the death of the parasite. Loukeris laboratory is interested on the identification and functional characterization of essential proteases since they are considered good targets for drug design. However, inability to disrupt the corresponding genes constitutes an obstacle to proteases further study and to the assignment of functional roles for those. An alternative to the traditional knock out approach has been investigated the last few years in our lab, which employs macromolecular protease inhibitors (MPIs) in order to functionally inactivate critical proteolytic machineries. Those MPIs are expressed in a stage- , time- specific manner in transgenic parasites**.**

In our laboratory *Toxoplasma gondii* tachyzoites are utilized in parallel with the rodent malaria parasite *Plasmodium berghei* since they share many similarities with Plasmodium merozoites and because this stage of *Toxoplasma* is genetically tractable. Furthermore, tachyzoites allows us for effective cell biology since its sub-cellular organelles are easily discerned**.**

One characteristic example of an apicomplexan protease with yet unassigned functional roles and substrates is Subtilisin 2 (SUB2). PbSUB2 is an essential protease for Plasmodium merozoites

which may be involved in juxta-membrane proteolysis (see Figure 2 depicted as yellow). Attempts to genetically disrupt the corresponding PbSUB2 gene were unsuccessful, although the genetic locus is accessible (19). Moreover, it was shown (20) that PbSUB2 is also expressed at the ookinete stage, during the invasion of the mosquito midgut epithelial cells by the ookinete, which implies an essential role of SUB2 in all invasion processes. In order to assign a functional role to SUB2 during mosquito midgut invasion a conditional silencing was attempted. Endogenous PbSUB2 promoter was replaced with that derived from the merozoite specific gene AMA1. In this way, it was expected that SUB2 activity would be available to merozoites, while it would be depleted from ookinetes. Although promoter replacement took place, SUB2 protease was detected in ookinetes indicating that stage specific regulation of SUB2 expression may involve additional to the promoter genomic control regions.

In an ultimate attempt to overcome the adversity to approach SUB2 function by any means the NcPI-S inhibitor derived from the related apicomplexan species *Neospora canninum* that was shown to specifically inhibit subtilisins *in vitro,* was employed (21). NcPI-S was expressed at the ookinete stage by placing it under the control of the strong ookinete specific CTRP promoter. The endogenous signal sequence of the NcPI-S was removed and NcPI-S was fused to the PbSUB2 signal peptide to ascertain that it would be trafficked within the secretory pathway of ookinetes. The fusion construct also contained the V5 epitope tag allowing protein detection. NcPI-S expressing ookinetes demonstrated a 97% decrease in oocyst formation; the oocysts were sporulation defective and the electron micrographs of NcPI-S expressing ookinetes demonstrated a deformed secretory pathway. In addition, ectopic expression of NcPI-S serpin in *Toxoplasma* tachyzoites under the strong constitutive promoter derived from the dense granule gene GRA1, resulted in a growth delay of transgenic tachyzoites in comparison to the wild type ones treated under similar conditions.

Scope of Master Thesis

The efforts to functionally inhibit proteases in apicomplexans by using NcPI-S serpin defined a novel strategy to generate genetically attenuated parasites, which is currently extended towards two directions in our laboratory. The first direction is to use molecularly engineered serpins in order to block proteolysis in invasion related organelles and the other is to test the inhibitory potential of additional naturally derived serpins against proteolytic machineries of apicomplexan parasites by using Plasmodium berghei ookinetes as a test tube system.

We decided to target molecular engineered MPIs in the micronemes of Toxoplasma tachyzoites aiming to inhibit critical proteolytic steps that take place in those organelles and we also attempted to develop a one step cloning transfection vector, which would facilitate one step sub-cloning of natural or molecular engineered MPIs and the subsequent generation of transgenic P. berghei parasites expressing those MPIs at the ookinete stage. In this case a high throughput screen would develop allowing us to identify serpins interfering with ookinete midgut invasion and ookinete to oocyst transition.

Project 1: Construction of one step cloning transfection vector for Plasmodium berghei.

In order for someone to make comparisons between the inhibitory effect of different types of serpins, it is needed to ascertain that components that determine expression levels, localization etc will remain the same. We can ascertain that the stage expression and the intensity of the expression will be the same among different PIs by retaining the same promoter. We can ensure that the PI will be localized at the same sub cellular organelles (Rhoptries, Micronemes) by placing in the construct the same localization signals. The stability of the m RNA s will be exactly the same when the same 3'UTR is retained.

The PI of our choice can be easily PCR amplified and sub-cloned in one step cloning transfection vector. Consequently, it is very easy to generate many different PI expressing strains and then test them in in-vivo.

We specifically chose the stage of ookinete for the expression of our PIs. The simplicity of the secretory pathway of the ookinete (only micronemes are present) makes it *easy* to compare the effectiveness of PIs.

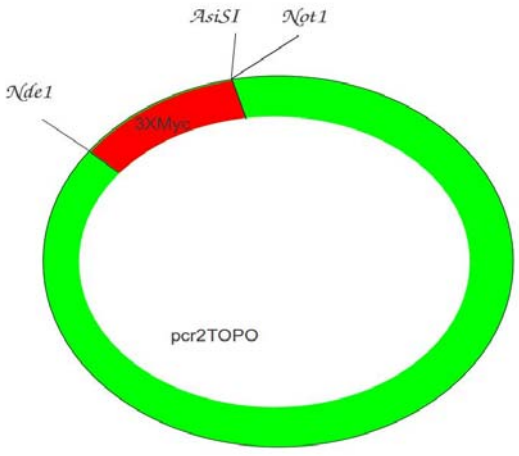
Plasmodium

Construction of the one step cloning transfection vector.

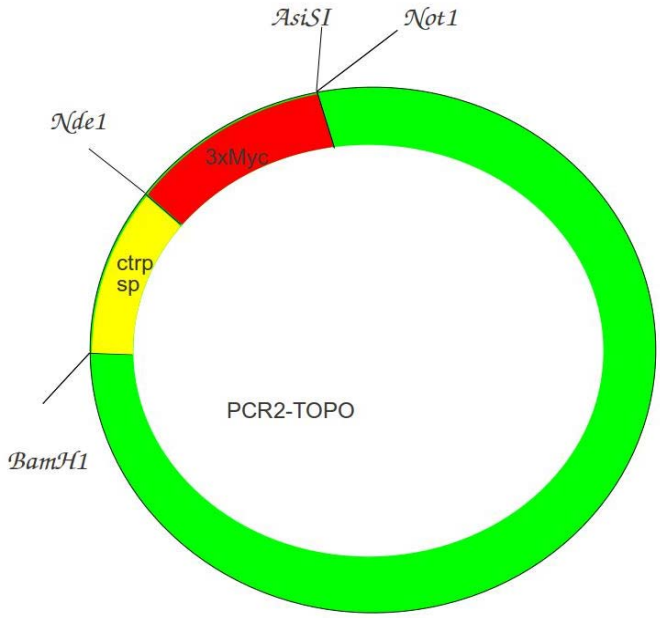
The 3xmyc tag was PCR amplified containing the Nde1 at the 5' prime and AsiSI, Not 1 at the 3' end. The PCR amplified construct was sub-cloned in the PCR2 p TOPO vector. The latter was digested with Nde1 and Not 1 restriction sites. 3XMyc tag was gel extracted and ligated to a proper vector containing the CTRP signal peptide (hereafter termed sp) that was also digested with Nde1 and Not 1 restriction sites. The latter vector was digested with BamH1 and Not1 restriction sites (hereafter termed R.S.) and the whole CTRSP-3Xmyc tag was subcloned to the nod32 vector that contained the ctrp promoter and the 3 ' UTR of Pbs21 m RNA. The whole sequence ctrp promoter -ctrp sp-3xmyc tag-3'UTR of Pbs21 was removed with the EcoR1 sites and sub-cloned to the EcoR1 digested dssu vector that contained the dssu locus.

This is a series of schematics that illustrate the sequential sub-cloning steps for the construction of the one step cloning transfection vector.

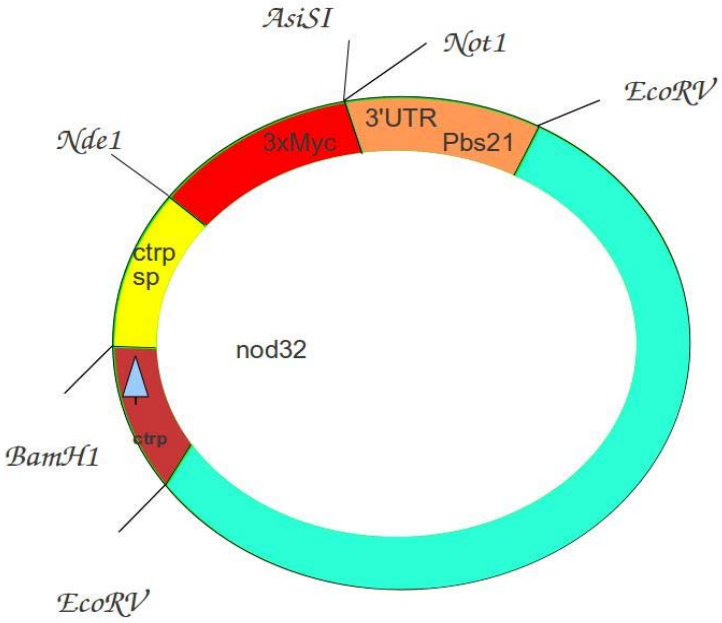
Step 1: PCR amplification of 3XMyc tag with Nde1 and AsiSI, Not1 R.S and sub-cloning in the p CR2-TOPO vector. FW primer: 5 '-CATATGGAACAAAAATTAATTTCTGAA-3' and REV: 5'GCGGCCGCGCGATCGCAAGATCTTCCTCTGAGAT-3'



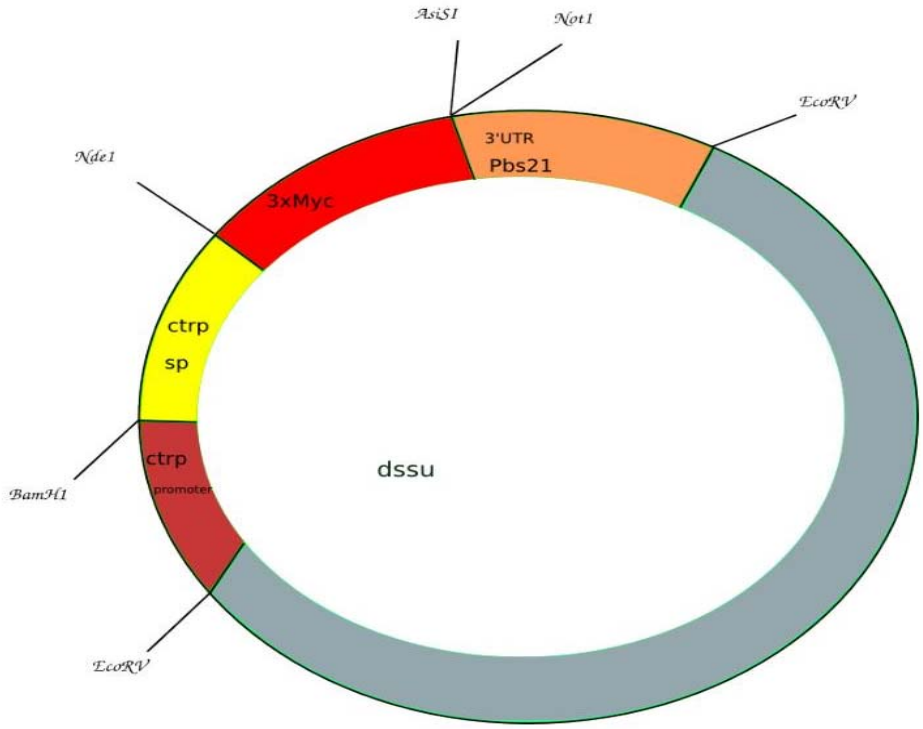
Step 2: Sub cloning of the 3XMyc tag in a p CR2-TOPO vector containing the ctrp sp using the Nde1 and Not1 R.S.



Step 3: Sub cloning of the ctrp sp-3xmyc in the nod32 vector containing the ctrp promoter and the 3'UTR of Pbs21 with the BamH1 and Not1 R.S.



Step 4: Sub-cloning of ctrp promoter-ctrp sp-3xmyc tag-3'UTR in dssu vector with EcoRV R.S. and ascertainment of the correct orientation using the Hind3 Restriction enzyme



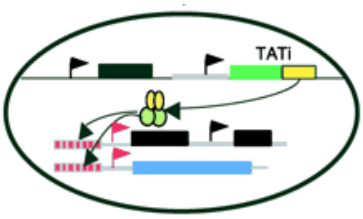
The one step cloning transfection vector will be validated through the sub-cloning of the NcPI-S in the vector. We expect our transfection vector to be efficient, and to demonstrate a decrease in the oocyst number, a deformed secretory pathway and the presence of sporulation defective oocysts. This will legitimately allow us to use the one step cloning transfection vector for several other PIs.

We reason that the one step cloning transfection vector is the first critical step in the development of a high-throughput assay that will collectively analyze and compare the inhibitory effects of several protease inhibitors.

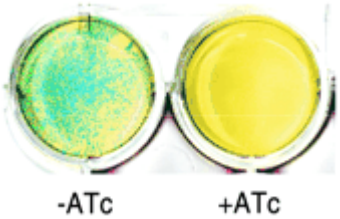
Project 2: Generation of plasmid constructions aiming to target serpins to micronemes.

Two different sequences are known, which efficiently target exogenous molecules into the micronemes of Toxoplasma tachyzoites when fused to them. One allows amino-terminal fusions and the other carboxy-terminal. The amino-terminal fusion sequence is the TgSUB1 pro-domain, which is shown to target TgSUB1 protease to the micronemes (22). The carboxy-terminus fusion sequence is the trans-membrane carboxy terminal domain (hereafter mentioned TMCTD) of MIC12 protein that encompasses a localization signal for micronemes (23). We used both sequences in order to generate fusions of NcPI-S, as well as, of an additional subtilisin inhibitor belonging to the Kazal family and deriving from the phytopathogen Phytophthora infestans.

Toxoplasma is the only apicomplexan for which an inducible expression system has been developed. This system allows transcription in the absence of anhydrotetracycline (hereafter termed ATC) , while it blocks it in the presence of ATC (15). The gene of interest is placed under the control of the 7tetOp (inducible promoter) (16). The whole construct is transfected via electroporation to a Toxoplasma strain called TAti strain. The TAti strain has the trans-activator, which binds to the 7tetOp, stably integrated in its genome. In the absence of anhydrotetracycline the trans-activator binds to the 7tetOp and drives the expression of the gene of interest (hereafter termed GOI) (16). We can retain the parasite by not adding ATC and we can observe the effect of gene expression by removing the ATC (16). In this way conditional expression of the gene of interest is achieved. In our case, the conditional expression would allow us to observe the debilitating effects of PIs in an on and off fashion. Since this effect might be toxic a conditional expression system is an ideal system to use.



Picture 6 : The transactivator is shown in green and yellow. It binds to the 7tetOp sequences, and drives the expression of the downstream genes. Blue box depicts the LacZ gene. Science 25 October 2002: Vol 298. Mo. 5594, pp 837-840



Picture 7: No LacZ expression can be detected in the presence of ATC, but can be observed in the absence of ATC. Science 25 October 2002: Vol 298. Mo. 5594, pp 837-840.

We first generated the 7tetOp-ROP1 –myc-EPI1-TMCTD. EPI1 inhibitor of Phytophthora infestans consists of two domains, one atypical kazal like domain and one typical kazal like domain (24). The atypical kazal like domain contains 4 cysteines that form 2 disulfide bridges (24). The typical kazal like domain contains 6 cysteines that form three disulfide bridges (24). The disulfide bridges in the typical kazal like domain are Cys 1- Cys 5, Cys 2- Cys 4 and Cys 3-Cys 6 (24). The bridges between the atypical kazal like domain are Cys 1- Cys 5, Cys 2- Cys 4 (24). In other words, the disulfide bridge Cys 3-Cys 6 is absent since both Cys are absent as well. The atypical kazal like domain was shown to inhibit both Subtilisin A and P69 Subtilisin with the use of in gel protease assays (24). The atypical kazal like domain and the typical kazal like domain referred as α and β respectively, were individually expressed, run on an SDS gel and then allowed to inhibit Subtilisin and P69 in BIO RAD zymogram buffer system (24). Since the whole EPI1 was able to inhibit proteolysis but the β domain was not, it follows that the inhibitory effect is caused solely by the α domain of EPI1 (24). In 7tetOp-ROP1 –myc-EPI1-TMCTD plasmid construction, EPI1 inhibitor has been fused to the carboxyterminal domain of MIC12 and tagged with a myc epitope.

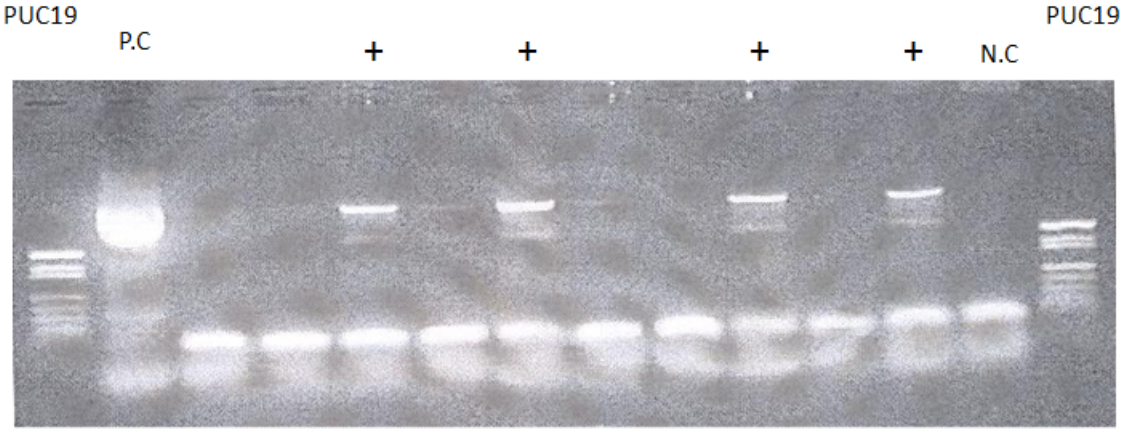
While this work was in progress, parallel efforts in our lab carried out by Zoe Tampaki demonstrated that indeed TMCTD domain fused to NcPI-S leads it efficiently into the tachyzoite micronemes. However, as a protease inhibition assay revealed the inhibitory potential of NcPI-S was deteriorated. Although these results may indicate a sensitivity of Kazal serpins in carboxyterminal fusions, myc-EPI1-TMCTD fusion differs from myc-NcPIs-TMCTD in the sense that between the inhibitory activity of EPI1 which resides in the domain a and TMCTD is inserted the typical Kazal domain b of EPI1, which may function as a stuffer. Testing in vitro the inhibitory potential of myc-EPI1-TMCTD using an established in the lab subtilisin Carlsberg assay will demonstrate if EPI1 may tolerate carboxyterminal fusions, while its expression in transgenic Toxoplasma tachyzoites will show its potential as an inhibitor of invasion related proteolytic processes.

The other fusion that we attempted was that of NcPI-S to TgSUB1 prodomain. The localization signal, TgSUB1 pro-domain has a unique feature. The TgSUB1 pro-domain can inhibit the TgSUB1 protease and we reasoned that this fusion to NcPI-S can increase the overall inhibitory effect of the engineered serpin.

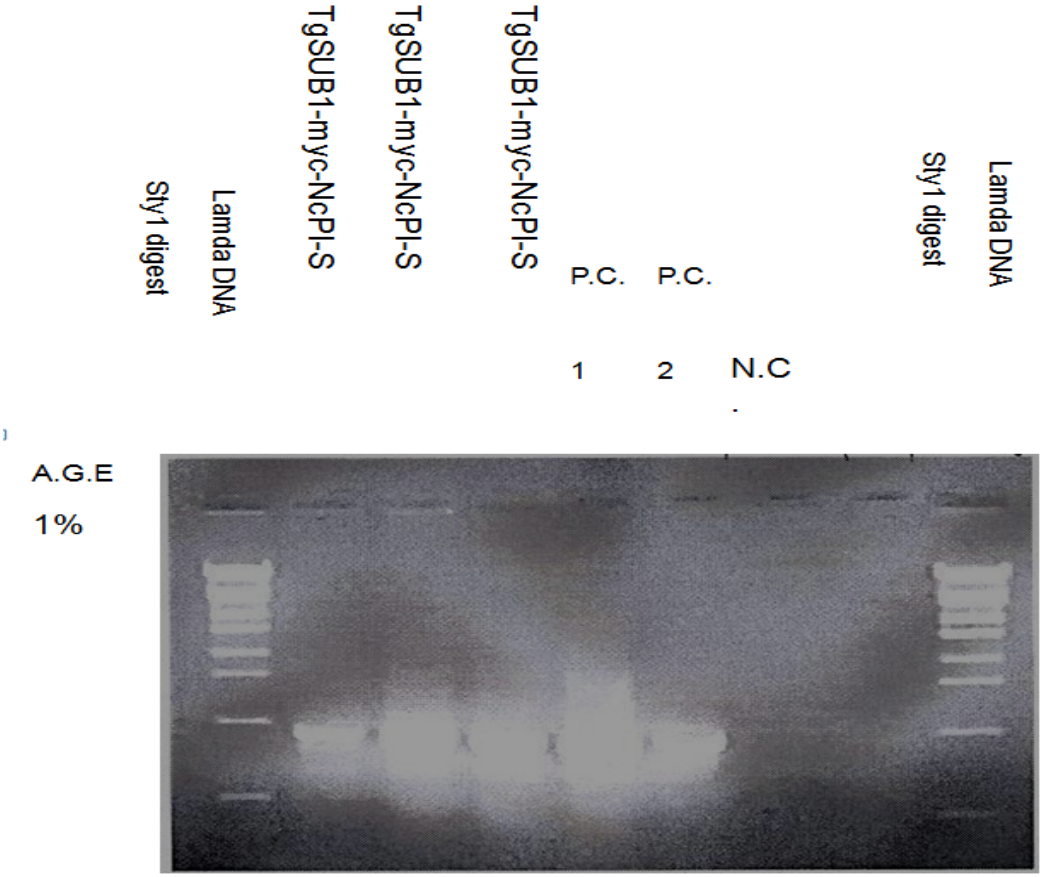
We attempted stable transfection for the 7tetOp-ROP1 –myc-EPI1-TMCTD and the 7tetOp-TgSUB1-myc-NcPI-S in repeated efforts, which is a strenuous procedure mainly because of the growth time requirements imposed by the parasite. We re-evaluated the procedure for the identification of positive clones and concluded that we necessitated a more rapid and accurate methodology for detection.

We named this methodology Toxoplasma PCR that facilitated the effective screening of the clones and served as an internal control by which we ascertained that the transfections were successful even though we had difficulties to observe protein bands of the expected sizes in our western blots. Specifically, for the 7tetOp-TgSUB1-myc-NcPI-S we also attempted transient transfections that were also proven successful, but we were also unable to observe the expressed protein.

Specifically, In the Toxoplasma PCR we were able to see bands of the correct size of the TgSUB1-myc-NcPI-S in many clones (884 bp), ascertaining that the stable transfection was successful. The positive Toxoplasma tachyzoites are represented with +, the positive control is abbreviated P.C and the negative control is referred as N.C.



The transiently transfected Toxoplasma TgSUB1-myc-NcPI-S tachyzoites that were also examined with Toxoplasma PCR demonstrated the distinctive band of the 884 bp length. Notice the presence of the TgSUB1-myc-NcPI-S band in the positive control and its absence in the negative control. The first positive control referred as P.C.1 is a plasmid containing the TgSUB1-myc-NcPI-S cassette. The second positive control referred as P.C.2 is a positive clone identified from the aforementioned Toxoplasma PCR. The negative control is referred as N.C. A.G.E stands for agarose gel electrophoresis.



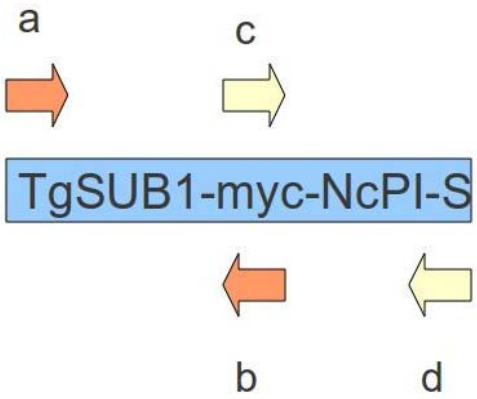
Our inability to observe any specific bands in the western blots of the Toxoplasma tachyzoites in both the NcPI-S and the EPI1 stably expressing tachyzoites under the control of the 7tetOp promoter, urged us to re-examine the cDNA sequence of our constructs for probable mutations. We were able to identify a frameshift mutation in the 7tetOp-TgSUB1-myc-NcPI-S and we decided to construct the 7tetOp-TgSUB1-myc-NcPI-S again. In parallel we also placed the corrected version of TgSUB1-myc-NcPI-S under the control of the strong GRA1 promoter derived from the gene encoding for the dense granule associated protein GRA1 (Gra- TgSUB1-myc-NcPI-S). Both constructs have been checked for mutations and none has been identified. We intend to perform both stable and transient transfections for both constructs, screen for positive clones with Toxoplasma PCR and finally grow a sufficient number of parasites for protein detection.

Toxoplasma

Single Overlap Extension PCR or S.O.E. PCR

In order to construct the TgSUB1-myc-NcPI-S cassette we used two pairs of primers. The primers are termed (a, b ,c, d). Primer a and b were used to PCR amplify the TgSUB1. The a FW primer contained the Nsi1 R.S. and the REV b primer contained the myc tag. The NcPI-S fragment was PCR amplified with the primers b, d. The FW c primer contained the myc tag and the REV d primer contained the Pac1 RS. The PCR products were analyzed on a 1% agarose gel electrophoresis. The two fragments were gel extracted following Qiagen Gel extraction manual's instructions. The gel extracted fragments were placed with the primers a and d and a third PCR was performed that resulted

in the generation of the TgSUB1-myc-NcPI-S fragment (Dr Zoe Tambaki). The PCR products were analyzed on a 1% AGE.



Sub-cloning of PCR amplified fragments in PCR2-TOPO.

The band was gel extracted and it was sub-cloned in the PCR2TOPO (Roche) vector following standard procedures. The ligation was verified with restriction enzyme diagnostic digestion (from hereafter R.E.D.D.) with EcoR1. The pTOPO-TgSUB1-myc-NcPI-S was digested with Nsi1 and Pac1 and was sub-cloned in the 7tetOp vector that was also digested with the Nsi1 and Pac1 restriction enzymes. The same was performed for the EPI1 containing the Fse1 and Sal1 restriction sites.

Sub-cloning of EPI1 and TgSUB1-myc-NcPI-S in 7tetOp Vector.

EPI1 was PCR amplified with the Fse1 and Sal1 RS towards the 5 'end and 3'end respectively (Primer design Dr Zoe Tambaki). The PCR amplified fragment was sub-cloned in the MIC vector which contains the MIC promoter, the signal sequence of ROP1, the myc epitope tag and the TMCTD of MIC12. The MIC vector was digested with Fse1 and Sal1 and the PCR amplified fragment, which was also digested with Fse1 and Sal1, was ligated to the MIC vector. The MIC vector containing the EPI1 was double digested with Nsi1 and Pac1 and the whole cassette containing the ROP1 signal sequence, the myc epitope tag, the EPI1 without the signal sequence and the TMCTD of MIC12 was ligated to the 7tetOp vector which was also digested with Nsi1 and Pac1. The TgSUB1-myc-NcPI-S was also double digested with Nsi1 and Pac1 and ligated to already digested 7tetOp vector with Nsi1 and Pac1.

Training in specific parasitic techniques.

Transfection of Toxoplasma tachyzoites

The transfection of 7tetOp-TgSUB1-myc-NcPI-S in TAti strain was performed using electroporation (Standard protocols: Toxoplasma Maniatis: Bootthroyd and Soldati). Experiments performed by Dr Zoi Tambaki. Briefly, the parasites that have lyzed a T175 flask are filtered in a polypropylene tube and their number is estimated through a haemocytometer. The parasites along with the plasmid DNA are resuspended in cytomix buffer inside an electroporation cyvette. For, the electroporation we use the BTX ECM 630 where the single pulse is 1,5 kv, resistance is set to 25 Ω and the capacitor is set to 25μF. Subsequently the parasites are transferred to a confluent T25 flask.

Cloning of Toxoplasma tachyzoites

The RH ^ HXGPRT TAti strain was passaged from 96 well to 24 well to T25 flask. In the final step there were two flasks for each clone. One flask was incubated with ATC and the other was not.

Maintenance of Toxoplasma parasites

The Toxoplasma tachyzoites were maintained in Dulbecco's medium that contained 10 % FBS, 1% penicillin /streptomycin and 1% glutamine.

Splitting of HFF cells

The splitting of HFF cells was performed following standard procedures (Cells: A Laboratory Manual, David Spector)

Harvesting of Toxoplasma tachyzoites

Toxoplasma tachyzoites were scraped, passed two times through a 18 ' gauge needle, once through a Millipore filter, resuspended in 10 ml PBS and centrifuged at 400 g at 4 ^{o} C for 15 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml PBS.

Western Blots

The eluate was resuspended in 2x SDS loading buffer, boiled for 2 minutes and allowed to cool in room temperature. The cellular extracts were analyzed in 17 % Resolving Gel and 5% stacking gel and then transferred to Nitrocellulose membrane (300 mA for 1hour and 20 minutes). Membranes were probed with anti Myc primary Ab (911b Cell Signaling Technology, Inc.) overnight in 1:20000 dilution (5% milk in TPBS) and with the secondary anti mouse HRP conjugated Ab (1: 10000) dilution (1% milk TPBS) for 1 hour. As a blocking buffer we used 5% milk. We used the Pierce ECL Western Blotting Substrate.

Toxoplasma PCR

This technique, that we called Toxoplasma PCR is based on a standard technique named Rapid

Characterization of cDNAs sub-cloned in Prokaryotic Vectors or more commonly colony PCR (Molecular Cloning :A Laboratory Manual, Third Edition).

In our variation of the technique the Toxoplasma tachyzoites that have lyzed an T25 flask of HFF cells are harvested, resuspended in PBS and the boiled for 5 minutes. Then we perform basic PCR with the primers amplifying the insert TgSUB1-myc-NcPI-S. Since the harvested tachyzoites were passed two times through 18 gauge needle and one time through filter, we exclude the possibility that besides the Toxoplasma tachyzoite's DNA there is also genomic DNA from HFF cells. Furthermore the NcPI-S gene is not found in the genome of the HFF cells.

1 μL from the genomic DNA of Toxoplasma tachyzoites was used for the identification of positive clones (clones carrying the TgSUB1-myc-NcPI-S) cassette stably integrated in the genome. In the PCR reaction we used the primers **a** (FW 5'CCCATGCATATGGGGTCATCTCAGCCATTG-3') and **d (**REV 5'TTAATTAAATGTTTTTGCTTGGATTTATTCC-3**')** that were described previously. Initial denaturation was set to 95o C for two minutes. The denaturation temperature was set to 95 ^{o} C for 30 sec, the annealing was set to 62 ^{o} C for 30 seconds, and the elongation was set to 72 ^{o} C for 52 seconds. The cycle was repeated 35 times and the final elongation was set to 72 ^{o} C for 4 minutes. The products of the Toxoplasma PCR were analyzed in 1, 5 % agarose gel electrophoresis.

Restoration of the correct O.R.F in TgSUB1-myc-NcPI-S cassette.

Our inability to observe any bands in the western blots ofTgSUB1-myc-NcPI-S tachyzoites urged us to search for possible mutations in our cassette. Unfortunately, we discovered a frameshift mutation in our cassette. Consequently, we followed a series of sub-cloning steps to alleviate the problem. NcPI-S with the correct ORF was removed from a vector of our lab and was ligated in the vector TgSUB1myc-NcPI-S that had been double digested with the R.S. Fse1 and Pac1 (N.E.B. enzymes). The Fse1 and Pac1 were flanking the NcPI-S. That cloning step restored the correct O.R.F of our cassette.

Construction of the Gra-TgSUB1-myc-NcPI-S vector.

We also decided to place the TgSUB1-myc-NcPI-S under the control of the strong Gra promoter. The cassette with the correct O.R.F. was double digested with Nsi1 and Pac1 (N.E.B. enzymes) R.S. and was ligated to the vector that contained the Gra promoter and had been digested with the Nsi1 and Pac1 R.S.

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